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Plants are valuable sources of medicinal compounds and their use for healing is well known from ancient times. Natural drugs obtained from plants represent about 25% of the prescription drug market in the United States. Plants have a long history of use in the treatment of various cancer types. Currently, 60% of the anticancer agents available in the market are derived from natural sources. Since phytoconstituents play a vital role in the discovery of various anticancer drugs, they have been chosen as the area of focus for our research. In this proposed study, four medicinal plants with reported anticancer activity were selected (*Hydrastis canadensis, Curcuma longa, Zingiber officinalis,* and *Alpinia officinarum*). All these plants were extracted by percolation and tested for anti-proliferative activity against *Dictyostelium* cells. *C. longa*, *Z. officinalis* and *A. officinarum* organic extracts all showed significant anti-proliferative activity in this preliminary bioassay. Of the three active extracts, the turmeric extract was chosen for further investigation because of its great historical significance and the promising results of recent phase I clinical trials. Using flash chromatography, a total of nine fractions were obtained from the complex *C. longa* organic extract. Curcumin in these fractions was identified and quantified using high performance liquid chromatography – electrospray ionization mass spectrometry (HPLC-ESI-MS). Other active components (demethoxycurcumin, bisdemethoxycurcumin and ar-tumerone) were also characterized using the same system. All these fractions were then tested for *in vitro* anti-proliferative activity against MCF-7 cells using the XTT assay to determine whether activity correlates

with the presence of curcumin in the fractions, or whether other (perhaps unidentified) compounds are involved. The results indicated that the major component curcumin was responsible for the majority of the anti-proliferative activity of the complex turmeric extract. Although no synergistic activity was seen for the various constituents present in the complex extract in this case, a novel approach for probing potential synergistic or additive effects was demonstrated. This approach could be applied to future investigations of synergistic or additive activity of medicinal plants.

CHEMICAL COMPOSITION AND ANTI-PROLIFERATIVE ACTIVITY OF SEVERAL MEDICINAL PLANTS

by

Siva Kumar Rapuru

A Thesis
Submitted to
The Faculty of The Graduate School at
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Greensboro

2008

Approved by

_____

Committee Chair

I dedicate my thesis to the most important people in my life.

To my parents Adi Lakshmi and Lakshmaiah Rapuru:

I thank you for giving me birth and taking all the pains to raise me into the person I am. I attribute my academic and professional success to you both. The pain and courage you showed in sending me abroad for my higher studies is indispensable. I am grateful to you for providing me financial and emotional support through out my career inspite of hardships you had in this journey. I love you so much.

To my lovely wife Rohini Devi:

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To my beloved brothers Narayana Rapuru and Anoj Rapuru:

You both are very special to me. I feel blessed to have you both in my family. My love and care for you both will stay forever. You are so far away from me and I miss you the most.

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To my in laws Krishnakumari and Srinivasa Chary Shesham:

Thank you for your love and care you showered on me. I always feel secured with the thought of having you in my life.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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ABBREVIATIONS

MTT: 3-(4, 5- Dimethylthiazol-2-yl)-2, 5- Diphenyltetrazolium Bromide

MCF: Michigan Cancer Foundation

MF: Molecular Formula

MW: Molecular Weight

C. longa: Curcuma longa

A. officinarum: Alpinia officinarum

Z. officinalis: Zingiber officinalis

et al;: and others

HPLC-ESI-MS: High Performance Liquid Chromatography - Electrospray Ionization - Mass Spectrometry

GCMS: Gas Chromatography Mass Spectrometry

C18: covalently bonded octadecylsilane

min: minutes

mL/min: milliliter per minute

µm : micrometer

µL: micro liter

m/z: mass to charge ratio

V : volts

kV : kilovolts

DMSO : dimethyl sulfoxide

NaCl : sodium chloride

KCl : potassium chloride

Na_2HPO_4: disodium hydrogen phosphate

KH_2PO_4: potassium dihydrogen phosphate

AX: axenic

cm: centimeter

rpm: rotations per minute

h : hours

M : molar

mM : millimolar

CO_2: carbon dioxide

EDTA: ethylene diamine tetra acetic acid

nm : nanometer

µM : micromolar

Avg : average

µg/mL : microgram per milliliter

Conc : concentration

Wt% : weight percent

Std. Dev : standard déviation

Org ext : original extract

Abs : absorbance

SD : standard déviation

approx : approximately

rpm : rotations per minute

CHAPTER I

INTRODUCTION AND RESEARCH GOALS

1.1 Natural products and their role in cancer treatment

Natural products are valuable sources of medicinal compounds and their use for healing is well known from ancient times. They still continue to provide new remedies to human kind in treating various diseases. Though significant development has been made in the combinatorial chemistry field, drugs derived from natural products still play an important role in the drug discovery process[1]. Today, natural drugs obtained from plants still represent about 25% of the prescription drug market in the United States[2]. Furthermore, according to the World Health Organization, 80% of the population in developing countries depends on traditional medicine for their primary health care, and 85% of traditional medicine is derived from plant extracts[2].

Cancer, a dreadful disease, is considered to be a leading cause of death in the United States, second only to heart disease[3]. Over the past 25 years, the United States government, through the National Cancer Institute (NCI), has expended billions of dollars to combat cancer[2]. In the process of development of drugs to treat cancer, natural products played an important role as contemporary cancer chemotherapeutic agents. Plants have a long history of use in the treatment of various cancer types[4]. Natural product chemists collect plants throughout the world, prepare their extracts and subject

them to various biological screening processes, which finally lead to the isolation and characterization of active compounds. Over 35,000 plant samples had been collected world wide by the National Cancer Institute (Natural Products Branch) and had screened around 114,000 extracts for antitumor activity[5]. As a consequence of extensive research, a number of clinically useful and market approved drugs are now available for use. It is a known fact that 60% of currently used anticancer agents are derived from natural sources[6]. Since plant constituents constitute an important source of cancer drugs, they have been chosen as the area of focus for this research.

1.2 Selection of medicinal plants

In this study, the first step was to select the medicinal plants which were shown to possess anticancer activity. To date, over 3000 species of plants had been reported to possess anticancer properties[7]. So, correct choice of plant material was necessary. Hence, in order to narrow the list of plants the following factors were considered:

- Plants that showed success with *in vitro* and/or *in vivo* studies
- Plants whose exact mechanism of action was not fully understood
- Plants that are native to North Carolina

Finally, four plants were selected and subsequently obtained to begin the preliminary research. Of these chosen plants, goldenseal is the only plant native to North Carolina.

1) Goldenseal (*Hydrastis canadensis*) – Rhizome

2) Turmeric (*Curcuma longa*) – Rhizome

3) Ginger (*Zingiber officinalis*) – Rhizome

4) Lesser galangal (*Alpinia officinarum*) – Rhizome

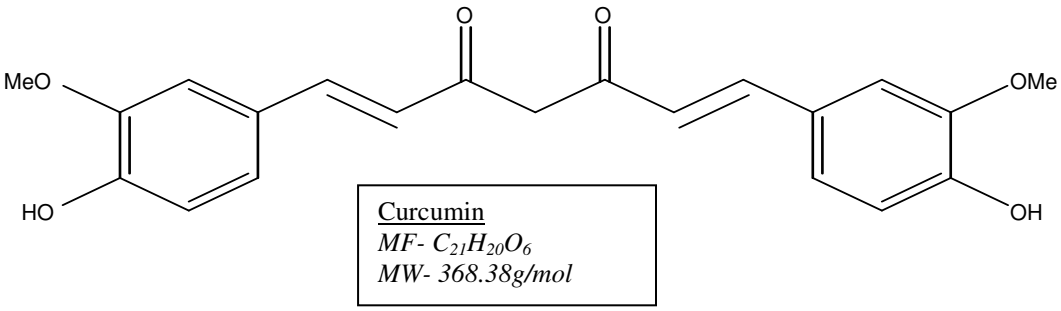
Of all the plants selected, turmeric, ginger and lesser galangal extract all showed activity in our preliminary bioassay. Of the three active extracts, the turmeric extract was chosen for more detailed studies given its traditional and clinical significance[8, 9]. Turmeric (*Curcuma longa*) has been used for thousands of years as a healing agent for variety of illnesses. Turmeric was found to be a vital source of active components that have strong anti-inflammatory and anticancer activities, including most notably the compound curcumin[10]. Based on several research studies, curcumin can be considered as a promising tool for cancer therapy and also has undergone a number of phase I human trials[11]. Although several cellular and molecular mechanisms of action for curcumin have been proposed the precise mode of anti-proliferative activity of curcumin has not been fully elucidated[12, 13]. Since turmeric extract is a mixture of several components, it is of interest to study how the presence of these multiple components affects the overall anti-proliferative activity of the complex turmeric extract. Taking all these facts into consideration, the turmeric plant was chosen as the plant of focus for this research.

1.3 *Curcuma longa* (Turmeric)

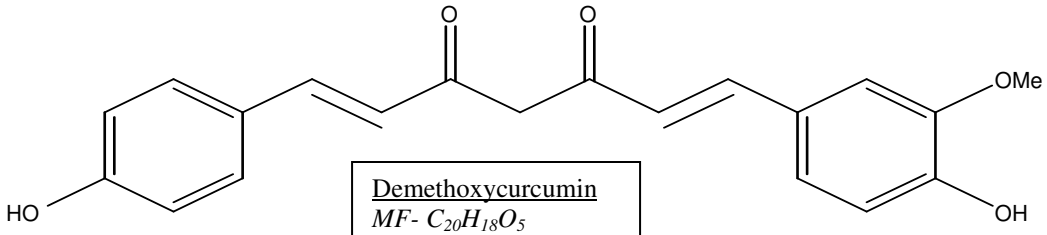
Curcuma longa or turmeric, a perennial herb belonging to ginger family, is a tropical plant native to southern and southeastern tropical Asia. The rhizome part of the turmeric plant is considered to be the main source of active components. The dried rhizome of turmeric contains the flavonoids curcumin, demethoxycurcumin and

bisdemethoxycurcumin, which are commonly known as curcuminoids, as well as volatile oils (mostly terpenoids) such as tumerones, atlantones and zingiberene[14, 15] (**Figures 1&2**). The most extensively studied component is curcumin (diferuloylmethane) which comprises 2-5% of the total components in turmeric. Over 2000 papers have been published on turmeric over the last 50 years[16].

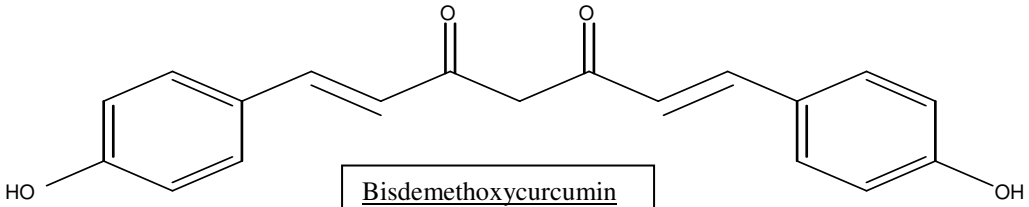
Curcumin possess various biological activities like anti oxidant, anti inflammatory, anti platelet, anti viral, anti fungal, and anti bacterial activities and also has been used in the treatment of Alzheimer's disease, multiple sclerosis and rheumatoid arthritis. Moreover, there is extensive literature which indicates the potential of curcumin in the prevention and treatment of various cancers[7]. Of all the curcuminoids, curcumin was found to possess the most potent anti-proliferative activity (e.g. human ovarian cancer cell line Ho-8910 inhibition by MTT assay[17]). In one study, it was shown that demethoxycurcumin is a better anti-proliferative agent than the other two curcuminoids (MCF-7 human breast cancer cell line inhibition[18]). Bisdemethoxycurcumin also showed more significant cytotoxic activity than the other two [19]. It was also found that all three curcuminoids exhibited similar anti-proliferative activity against various cell lines (Jurkat, KBM-5, and A549 cell lines[16]). Among the volatile oils, ar-tumerone exhibited potent cytotoxic effect on cancer cell lines like K562, L1210, U937 and RBL-2H3 by MTT assay[20].



Curcumin
MF- $C_{21}H_{20}O_6$
MW- 368.38g/mol



Demethoxycurcumin
MF- $C_{20}H_{18}O_5$
MW- 338.35g/mol



Bisdemethoxycurcumin
M.F- $C_{19}H_{16}O_4$
M.W- 308.32g/mol

Fig 1 - *Structures of curcuminoids found in turmeric extract*

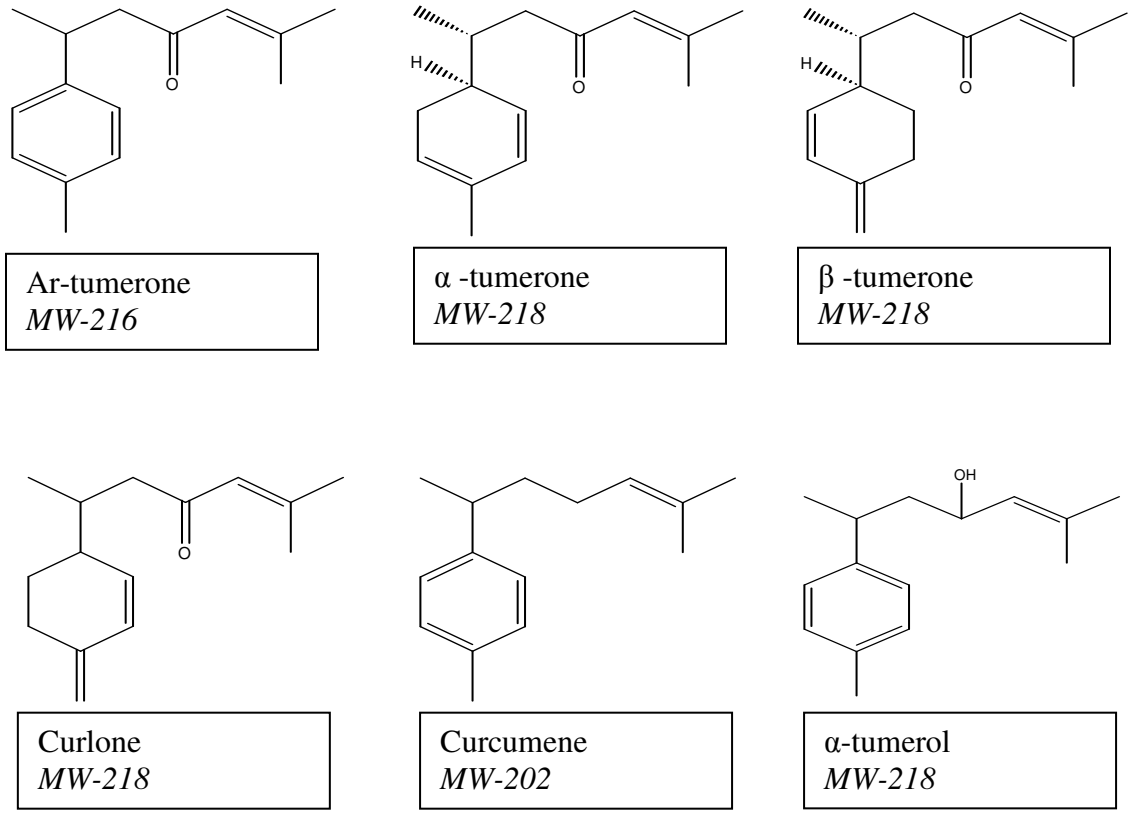


Fig 2- *Structures of main volatile oil components present in Turmeric oil*

1.4 Anticancer activity of curcumin *in vitro*

Curcumin, an active constituent of *C. longa* extract, has the ability to induce apoptosis in different cancer cells. This property was used in developing curcumin as a universal cancer prophylactic agent[21]. Curcumin has been shown to suppress transformation, proliferation and metastasis of tumors through various mediators like transcription factors, growth factors, growth factor receptors, inflammatory cytokines, protein kinases, various enzymes and genes regulating apoptosis and proliferation[22]. It also inhibits proliferation of cancer cells by arresting them in various phases of the cell

cycle and by inducing apoptosis, indicating its potential in the treatment of various cancers. *In vitro* studies conducted on curcumin have shown to inhibit the growth of large number of human cancer cell lines, like leukemia, prostate, lung, renal, colon, melanoma, breast[23], hepatocellular and ovarian carcinomas.

1.5 Methods used for the analysis of Turmeric extract

The main aims of natural product research are - qualitative and quantitative analysis of medicinal plant constituents and isolation of biologically active purified plant extract fractions or compounds. So, considering the fact that a single plant can have several thousand constituents, the above goals can be achieved by employing rapid and high performance separation methods. A major task for a phytochemist in the analysis of natural compounds is the characterization of compounds with minimal sample preparation. Usually compounds present in the plant extracts can be separated rapidly using chromatography. A liquid chromatographic method with spectrophotometric detection was developed for the first time in 1953 to separate and quantify curcuminoids[24]. Later on, various methods such as paper or thin layer chromatography[25, 26] (TLC), capillary electrophoresis[27] (CE), gas chromatography[28] (GC), high performance liquid chromatography (HPLC), and its coupling to photodiode array detection (HPLC-DAD[29]) and mass spectrometry (LC-MS[30]) were used to analyze the chemical constituents of turmeric extracts. Xian-Guo He *et al*. (1998) were the first researchers to employ online high performance liquid chromatography-electro spray ionization-mass spectrometry (HPLC-ESI-MS) for the identification of turmeric constituents[30]. Not only

individual curcuminoids but also a number of essential oil constituents like ar-tumerone and curlone could be identified from a single HPLC analysis. Hongliang Jiang *et al.*(2006) and Kuo-Yi Yang *et al.*(2007) validated HPLC coupled with tandem mass spectrometry (LC-MS/MS) to identify curcuminoids in turmeric extract[31, 32].

The aroma of turmeric is due to the presence of several volatile oils. These volatile oils are usually a mixture of sesquiterpene ketones and alcohols. Malingre (1975) for the first time reported volatile oil constituents' ar-tumerone, tumerone, curlone, curcumene and other sesquiterpene alcohols from *Curcuma longa*[33]. Phan *et al*. (1987) for the first time reported the GC-MS analysis of turmeric oil[34]. Richard Hiserodt *et al.* (1996) employed direct thermal desorption-gas chromatography-mass spectrometry (DTD-GC-MS) for the analysis of various volatile and semi-volatile compounds in powdered turmeric[35]**.** GC-MS serves as a convenient and sensitive analytical technique for analyzing essential oils, and remains one of the most common methods applied for this purpose. And also online HPLC-ESI-MS technique was used to analyze various sesquiterpenoid volatile components present in turmeric extract[30, 35].

1.5.1 HPLC-ESI-MS

Chromatographic separation coupled with mass spectrometric detection provides a pathway for the rapid and sensitive characterization of active compounds from plant extracts. LC-MS, which couples liquid chromatography (LC) with mass spectrometry (MS), has gained attention as a convenient method for identification, structural determination, and quantitative analysis of various bioactive compounds in complex

extracts. LC-MS is considered to be a major breakthrough in the analysis of natural products possessing significant biological activities. Of the various LC-MS techniques, high performance liquid chromatography coupled to electro spray ionization-mass spectrometry (HPLC-ESI-MS) is an ideal technique for the analysis of a wide variety of analytes[36]. This soft ionization technique coupled to a single quadrupole mass spectrometer provides the molecular weight of compounds, and further structural details can be obtained with HPLC-MS-MS systems (those with triple quadrupole or ion trap mass analyzers), which provide information on the characteristic fragmentation pattern typical of a compound. In contrast to other ionization techniques, multiply charged analytes can easily be formed with ESI, making it possible to analyze larger molecules. Small polar to medium polar analytes are also readily ionized with this technique, making HPLC-ESI-MS useful in phyto-chemical investigation[37]. In most quantitative HPLC-ESI-MS methods, low detection limits and reproducible results are the desirable features. A schematic of an HPLC-ESI-MS system is shown in **Figure 3**. A typical HPLC-ESI-MS system has mainly three different regions; the HPLC system, the ESI ion source and the mass spectrometer. An HPLC-ESI-MS system similar to that shown in Figure 4 was employed for this study to analyze the plant extracts.

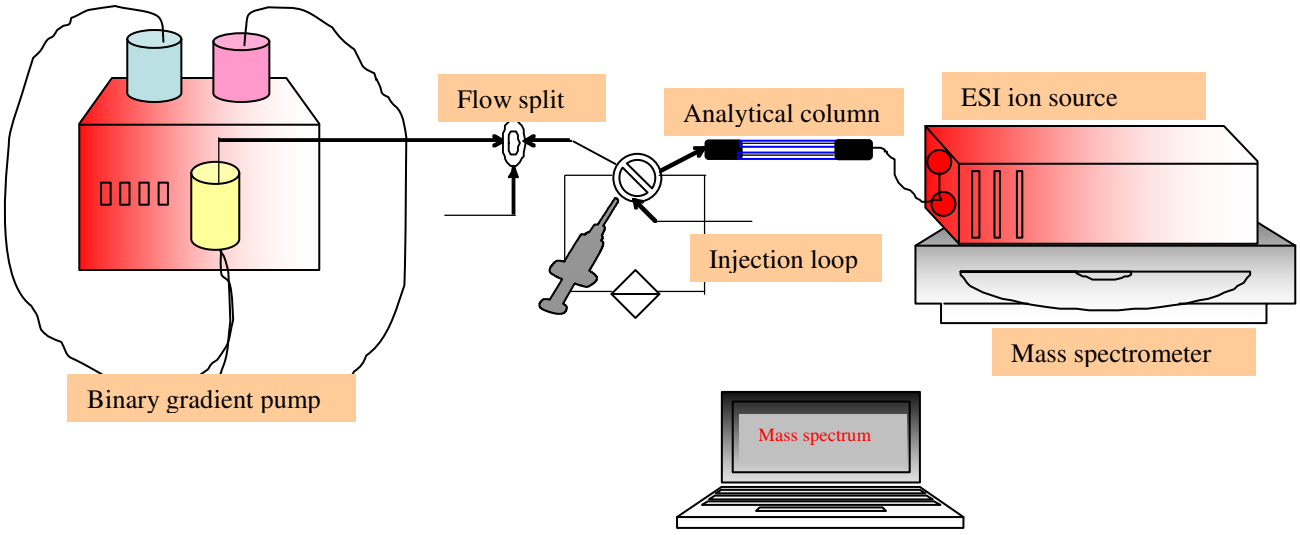


Fig 3 - *Schematic representation of an HPLC-ESI-MS hyphenated system*

1.6 Evaluation of biological activity (Anti-proliferative activity)

The isolation of anticancer natural products needs an appropriate bioassay to guide fractionation at each step. Many bioassay systems are available for anticancer drug discovery. A screening bioassay is applied to large number of initial samples to identify those with desired bioactivities. The purpose of using this bioassay is to discard inert materials and identify active fractions. A monitoring bioassay is used to guide fractionation of crude materials towards isolation of the pure active components. Modern analytical instrumentation may be most effective in bioassay-directed isolation. In this research, *Dictyostelium* cells and MCF-7 human breast cancer cells have been selected to test for anti-proliferative activity of fractions and constituents of *C. longa*.

1.6.1 *Dictyostelium* cells

In this study, the initial screening of all the plants extracts was done using the model system *Dictyostelium discoideum*. *Dictyostelium* was used as a powerful eukaryotic model system for identifying and characterizing the basic mechanisms which control the cell division[38], cell migration[39], multicellular development processes[40] and other vital cellular processes that play significant roles in various diseases. When compared to other cell types, this cell type is considered to be less complicated (i.e. easy to grow, handle and manipulate). Several studies on *Dictyostelium* have provided useful information on how the various cellular processes were been altered during the uncontrolled cell multiplication seen in tumor growth[41, 42]. This model system allows identifying a specific gene involved in this process and also serves as a model for processes that occur in mammalian cells, since it has some of the complex features that resemble mammalian cells. Perhaps most importantly, *Dictyostelium discoideum* is an extensively studied model organism for which a great deal of genome sequencing work has been completed[43].

1.6.2 MCF-7 Human breast cancer cells

In addition to *Dictyostelium* cells, the MCF-7 Human breast cancer cell line was employed in order to identify the fractions of *C. longa* with significant anti-proliferative activity. The MCF-7 cell line, initially developed by Soule et al. (1973), was used universally for various *in vitro* and *in vivo* studies on potent compounds used in the

therapy of breast cancer as it relates to the vulnerability of the cells to apoptosis[21]. This cell line is the best characterized of all the existing estrogen responsive lines. Since MCF-7 cells are mammalian cells, the data derived from these cell lines will be used as a first step towards gaining insight into the possible anti-tumor activity of the components of interest. Follow up experiments would be necessary to confirm the *in vivo* relevance of any anti-proliferative effects observed *in vitro*. Curcumin has already been shown to suppress cell proliferation of MCF-7 human breast cancer cells by targeting several molecules and pathways involved in cancer pathogenesis[44, 45].

1.7 Measuring viability of cells using XTT assay

In the process of screening various cytotoxic agents, determination of viable cell number is often used to measure the cell proliferation rate. The XTT assay, a colorimetric method, is a valuable method for screening of plant extracts for anti-proliferative activity. The XTT method is a simple, fast, rapid and accurate assay and yields reproducible results[46]. In this assay, the XTT reagent (tetrazolium salt) combines with mitochondrial dehydrogenase enzyme of viable cells yielding orange colored formazan crystals. The amount of formazan formed indicates the degree of cytotoxicity caused by the test samples (i.e. the amount of formazan resulted from XTT is proportional to the total number of living cells in the sample wells). The resulting orange colored product was quantified by measuring the absorbance spectrophotometrically. The percentage of cells surviving after the sample treatment is determined by comparing the absorbance of treated cells with that of the control cells. Since dead cells are unable to metabolize

tetrazolium salts, the colorimetric assay like XTT can be used to measure cell viability/cell proliferation rate. Apoptosis, normal cell death, requires metabolism of cells. Hence for this reason colorimetric assay (XTT), which can not measure cellular damage, can detect cell death only at later stages of apoptosis when the cellular metabolism is reduced.

1.8 Research Goals

The main objective of this study is to separate and quantify phytoconstituents from *Curcuma longa* (Turmeric) plant extract and then test the bioactivity of isolated compounds. Turmeric extract is a complex extract with several components which have significant biological activities [16, 12 & 47]. Though significant research was done on all the individual components of the turmeric extract, still the relative importance of all these components for the overall anti-proliferative activity is not clear. It is also not known whether there are compounds other than curcuminoids and volatile components that are important in the anti-proliferative activity of *C. longa*. The major goal of this study is to address this gap in the knowledge base. The main aim of this research is to study how the various components present in the complex turmeric extract contribute to its anti-proliferative activity. To accomplish this goal, isolation, identification and quantification of constituents from the turmeric extract will be accomplished using HPLC-ESI-MS. Further, preliminary screening experiments will be conducted on the plant extracts obtained using *Dictyostelium* cells to test for anti-proliferative activity. Once the complex turmeric extract is separated into individual fractions or compounds, these individual

fractions will again to be tested for anti-proliferative activity against MCF-7 human breast cancer cells using the XTT assay. The flow chart (**Figure 4**) shown below represents an outline of this research-

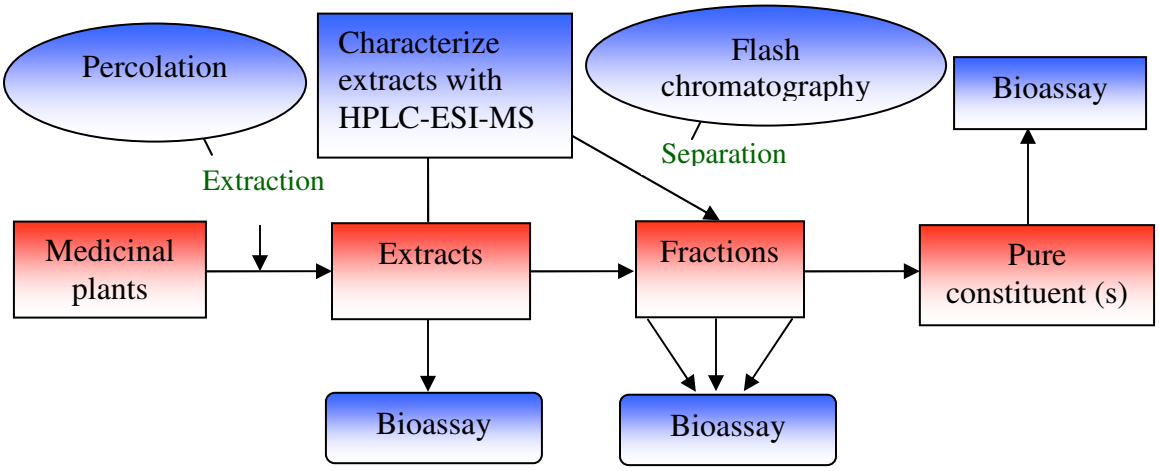


Fig 4 – *Flow chart detailing various steps involved in the research*

CHAPTER II

EXPERIMENTAL METHODS

The following section gives a brief overview of the experimental procedures used in the research. In order to identify and quantify active components from plant extracts the first step would be to extract these components using a suitable extraction process. Percolation utilizing suitable solvents was employed to obtain a complex extract of all four plants- *Hydrastis canadensis, Curcuma longa, Zingiber officinalis, and Alpinia officinarum*. These extracts were analyzed using HPLC-ESI-MS to identify major constituents of the extracts. The anti-proliferative activity of these extracts was then tested using the *Dictyostelium* cell model. Flash chromatography was used to further separate the complex turmeric extract into fractions. The concentrations of the active components in the fractions obtained were also determined using HPLC-ESI-MS. Finally, the turmeric fractions were tested against MCF-7 human breast cancer cells to identify fractions with significant anti-proliferative activity and to correlate the HPLC-ESI-MS data with biological efficacy.

2.1 Extraction of *Curcuma longa*, *Hydrastis canadensis*, *Zingiber officinalis* and *Alpinia officinarum* plants

Two crucial stages involved in the isolation of phytoconstituents are the selection of plant material and the procedure of extraction. This includes grinding of the plant material, extraction of the constituents, and concentration and drying of the extracts. In this research, all the medicinal plants selected were extracted by percolation, which is a simple maceration process. Maceration is the most commonly used preparative extraction method by most of the phytoanalysts[48]. Using a laboratory grinder, the dried plant material was ground to fine powder to create as much surface area as possible. The amounts of the ground plant material taken for the purpose of extraction are given in **Table 1**.

Table 1 – Amounts of ground plant material used for extraction

Plant	**Amount of ground plant material**
Goldenseal (*Hydrastis canadensis*) [*Pacific botanicals, Lot no.- 0906M-TCP*]	500g
Turmeric (*Curcuma longa*) [*Horizon Herbs, Lot no. – 4360*]	390g
Ginger (*Zingiber officinalis*) [*Horizon Herbs, Lot no. – 4361]*	416.5g
Lesser Galangal (*Alpinia officinarum*) *[Horizon Herbs, Lot no.- 4362]*	312g

2.1.1 Procedure

The ground plant material was initially submerged in the solvent mixture of dichloromethane – methanol (1:1) completely. The plant material was then left in contact with the solvent mixture overnight and the solvent was drained off the next morning. Next, the extracted plant material was covered in methanol and a second extraction was performed in order to rinse off the residual dichloromethane (approx. 6h). Then the methanol solvent was removed by suction filtration. Next, the dichloromethane – methanol (1:1) solubles were combined with the methanol solubles and then dried by rotary evaporation. Finally, sufficient water was added to cover the remaining plant extract, making a second extract (aqueous), which was then stored at -20°C. These procedures resulted in an organic solvent extract and aqueous extract for each of the four plants that could then be further analyzed using HPLC-ESI-MS and tested for bioactivity.

2.2 Identification of the active components from plant extracts using HPLC-ESI-MS

Once the final crude extracts are obtained, they have to be chemically screened for the presence of active constituents. The characterization of active metabolites in complex plant extracts requires sophisticated hyphenated techniques, which should provide good sensitivity and selectivity as well as structural information on the constituents of interest. For this study, the HPLC-ESI-MS technique was employed to identify the bioactive components of the plants extracted.

Initially, three concentrations (1mg/mL, 0.1mg/mL, and 0.01mg/mL) of both the organic and aqueous extracts were prepared using methanol solvent. Using a 0.2 µm nylon filter, all the samples prepared were filtered prior to analysis.

2.2.1 Instrumentation

Analyses were conducted using an Agilent 1100 series HPLC instrument with a C18 reversed phase column (Alltech, 50mm x 2.1mm, 3µm particle size) coupled with an ESI-ion trap mass spectrometer (LCQ Advantage, ThermoFinnigan). A 0.5µm pre-column filter (Mac Mode Analytical) attached to the column inlet was used to remove any residual particulate present in the HPLC solvents or samples. A gradient elution (0-4 min - 90% A, 4.00-30.00 min -90-0% A, 30.10-36.00 min – 90%A) was run with mobile phase solvents A (1% acetic acid in nanopure water) and B (HPLC grade acetonitrile) with a flow rate of 0.2 mL/min and injection volume of 10µL. The mass spectrometer was operated with a scan range of 50-2000 m/z, a capillary temperature of 275°C, and a sheath gas pressure of 20 (arbitrary units). Two analyses were carried out for each sample, one in the positive ion mode and one in the negative ion mode. The source, capillary, and tube lens voltages for the positive ion mode analyses were 4.5 kV, 3V and -60V, respectively, and for the negative ion mode analyses were 4.5 kV, -10 V and -50 V, respectively. The total analysis time was 36 min.

2.3 Primary screening of plant extracts *in vitro* (Anti-proliferative activity) using *Dictyostelium* cells

Once the extracts were prepared and characterized, they were initially screened for anti-proliferative activity using *Dictyostelium* cells. The purpose of this bioassay was to identify the crude plant extracts with significant inhibitory effects on cell growth *in vitro*. Three concentrations (2 mg/mL, 1 mg/mL and 0.5 mg/mL) of both the organic and aqueous extracts of all the plants were prepared using sterile technique in DMSO and phosphate buffered saline (PBS) solution [NaCl (81%) + KCl (2%) + Na_2HPO_4, dibasic (14%) + KH_2PO_4, monobasic (3%)] of pH 7.4. These experiments were accomplished under the direction of Dr. Paul Steimle and were performed by his student Erica Fields in the Department of Biology at the University of North Carolina, Greensboro.

2.3.1 Procedure for screening with *Dictyostelium*

Dictyostelium discoideum AX2 strain[49], an axenic mutant, was used for this *in vitro* cell inhibition assay. These cells were grown to confluence in 15 cm Petri dishes containing HL5 culture medium and were harvested by centrifugation. These cells were then used to prepare 5 $x10^4$ cells/mL cultures which were transferred into Falcon tubes. These cell cultures were then treated with different plant extract samples. All these cultures were grown with shaking at 185 rpm at 21°C. A positive control (lacking plant extract sample) for normal cell growth was also grown. All the cultures prepared contained 0.7% DMSO. An aliquot of each culture was removed at 24 h interval (up to 120 h) and the cells were counted using a hemacytometer. Growth rates for different

cultures were then plotted as cell number versus time. The composition of the HL5 culture medium is given below (**Table 2**).

Table 2 - HL5 culture medium composition (1liter)

1.)	5g Protease peptone
2.)	5g Thiotone E peptone
3.)	10g Glucose
4.)	5g Yeast extract
5.)	0.35g Na_2HPO_4. $7H_2O$
6.)	0.35g KH_2PO_4

All the ingredients were mixed to a final volume of 1 liter. The pH of the medium was adjusted with HCl to 6.4 - 6.6 and was then finally autoclaved.

2.4 Separation and quantification of curcumin from turmeric extract

The main goal of this experiment was to separate curcumin from the *C. longa* organic extract and further quantify the turmeric fractions containing various amounts of curcumin using suitable methods. All the fractions obtained must then be screened using a suitable assay of anti-proliferative activity.

2.4.1 Procedure

Flash chromatography over silica gel (Column dimensions, 15 × 1.5 cm) was employed to separate the complex *C. longa* organic extract. Using a binary solvent mixture of hexane and diethyl ether, a total of nine fractions of turmeric extract was obtained. The fractions were run in the increasing amounts of diethyl ether (10%, 20%, 30%, 40%, 50%, 60%, 70%, 85% and 100%). Each of the fractions obtained was aliquoted (1 mL) into a pre weighed centrifuge tube and the solvent was removed under vacuum to determine the dry weight. The dried samples were redissolved and diluted using methanol solvent to make three concentrations of each fraction (1mg/mL, 0.1mg/mL and 0.01mg/mL, expressed as mg dissolved solids per mL of solvent). Similar samples for the original turmeric extract were also prepared.

The concentration of curcumin present in the turmeric fractions were quantified using a standard of curcumin (Acros organics, New Jersey, USA). Stock solutions of this standard were prepared in methanol solvent at concentrations of 0.01M and 0.03M and then serially diluted to make standards of concentrations 1.0×10^{-4} M, 1.0×10^{-5} M, 1.0×10^{-6} M, 1.0×10^{-7} M, 3.0×10^{-5} M and 3.0×10^{-6} M. Finally, all of the standards, along with the turmeric fraction samples and the original extract samples were analyzed using HPLC-ESI-MS method (negative ion mode scanning) with the same conditions as described in Section 2.2.

2.5 Identification and quantification of ar-tumerone in the turmeric fractions using HPLC-ESI-MS

The complex turmeric extract has several volatile oil components besides curcuminoids. Among the volatile oil constituents, ar-tumerone, α-tumerone and β-tumerone have been shown to induce apoptosis on various cancer cell lines[16]. Of these three volatile oils ar-tumerone is the major component and is the most extensively studied compound. So, the next step in this research would be to identify and quantify ar-tumerone present in the turmeric fractions obtained. For this purpose HPLC-ESI-MS was utilized with the same parameters mentioned in Section 2.2. Three different concentrations (1mg/mL, 0.1mg/mL and 0.01mg/mL) of all the nine fractions including the original extract were made using methanol solvent. In order to quantify ar-tumerone present a standard ar-tumerone (Chromadex, Santa Ana, California, USA) was used. A stock solution of 0.01 M ar-tumerone was prepared in methanol and then serial dilutions of this solution were performed to prepare solutions with concentrations of 3.0×10^{-3} M, 3.0×10^{-4} M, 3.0×10^{-5} M, 3.0×10^{-6} M and 1.0×10^{-6} M. All these samples prepared (the fractions and the standards) were then analyzed using HPLC-ESI-MS in the positive ion mode.

2.6 Testing turmeric fractions for anti-proliferative activity against MCF-7 human breast cancer cells (XTT assay)

Once the major bioactive components present in the turmeric fractions were identified they had to be tested for *in vitro* anti-proliferative activity using a suitable cell

proliferation assay. For this purpose, the MCF-7 human breast cancer cell line was selected. The anti-proliferative activity of the complex turmeric extract, the turmeric fractions and the standard curcumin was tested against the MCF-7 human breast cancer cell line using the XTT assay in a 96 well plate format[50]. The purpose of using MCF-7 cells for this cell viability assay is that these cells are more amenable to the 96 well plate set up and also the results obtained from these cells should be more closely relevant to humans.

The first task in this assay was to determine the LD_{50} (median lethal dose) for pure curcumin which was determined by testing different concentrations of standard curcumin (1µM, 3µM, 10µM, 30µM and 100µM of curcumin in 0.5% DMSO in medium) on MCF-7 cells. Once the LD_{50} of curcumin was determined, turmeric fractions including the original extract containing varying concentrations of curcumin were tested against MCF-7 cell line, with the ultimate goal of determining which constituents of the fractions contributed to their anti-proliferative activity.

2.6.1 Preparation of Stock Solutions

For determining the LD_{50} of pure curcumin a stock solution of 24,000µM (100% DMSO) of standard curcumin was prepared. This stock solution was further diluted with media (without phenol red) to make a solution of 1200µM (5% DMSO) of standard curcumin. From this stock, serial dilutions were further made using media (5% DMSO) to make solutions of five different concentrations (1000µM, 300µM, 100µM, 30µM, and 10µM). Finally, 20µL of each of these concentrations when present in a final well

volume of 200µL represents a final concentration of 100µM, 30µM, 10µM, 3µM, and 1µM of standard curcumin (0.5% DMSO) in each well of the 96 well plate.

Stock solutions for all the turmeric fractions (except fraction 1) including the original extract were prepared based on the curcumin content present in the solid extracts. Initially a stock solution of 7000µM (100% DMSO) of curcumin for all the turmeric fractions including the original extract was prepared. This stock was further diluted with media (without phenol red) to make a 350µM curcumin (5% DMSO) stock solution. From this stock solution, serial dilutions were made with media (5% DMSO) to make solutions of two different concentrations (300µM and 30µM of curcumin). Finally, 20µL of each of these concentrations in a final well volume of 200µL yields a final concentration of 30µM and 3µM of curcumin (0.5% DMSO). For fraction 1, which has only ar-tumerone but not curcumin, the stock solution was prepared based on the amount of ar-tumerone present in the solid extract. All these solutions were prepared under sterile conditions.

2.6.2 Procedure

MCF-7 human breast carcinoma cells (ATCC), grown as a monolayer in 75cm^2 canted neck flask (Corning), were maintained in 500 mL Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 57 mL of 10% fetal bovine serum (FBS) (GIBCO), 5.8 mL of 1mM sodium pyruvate (Sigma-Aldrich), 5.8 mL of 2mM L-glutamine (Sigma-Aldrich), 5.0 mL non essential amino acids (Sigma-Aldrich), 500 µL insulin (Sigma-Aldrich) and 5.0 mL of 100mM penicillin/streptomycin (Sigma-Aldrich)

in a humidified atmosphere of 5% CO_2 at 37°C. Once they have reached 70-80% confluence, the culture medium was removed and the adherent cells were washed with phosphate buffered saline (PBS, pH-7.4) (GIBCO) solution. To loosen the cells from the flask surface, 0.5% Trypsin-EDTA (3mL) (GIBCO) was added and was diluted with 4 mL DMEM medium (with phenol red). From this stock culture of cells, cells of density 1×10^5cells/mL were prepared using DMEM medium (without phenol red) and were plated into each well (180µL) in a 96 well plate (Costar 3599, Corning, NY, USA). These cells were then incubated (at 37°C and humidified 5% CO_2 atmosphere) for 2-3 days until they reached 70% confluence. Subsequently 20µL of each sample dissolved in 0.5% DMSO in medium was added to each well (in triplicate). DMSO (0.5%) was used as vehicle control. Wells containing cell culture medium alone, cells in medium without sample (negative control), and the samples in medium alone at each concentration to be tested (blank control) were used as additional controls. The plate was shaken using a rocking shaker (Lab-Line Instruments Inc, Melrose Pk, IL, USA) with a speed of 4 rpm to ensure that the samples were thoroughly mixed into the media. Cells were then incubated for 72 h at 37°C and humidified 5% CO_2 atmosphere. Finally, 50µL of XTT reagent (Sigma Aldrich) (sodium salt of 2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide + 1% phenazine methosulfate (PMS), 5mg in serum vial) dissolved in cell culture medium with out phenol red and serum (5mL) was added to each well of the 96 well plate and then incubated for 6 h to allow the XTT to be metabolized. Using a rocking shaker (speed of 4 rpm) the plate was shaken to evenly distribute the dye in the wells. The final volume of each well was 250µL. The XTT reagent combines with

the mitochondrial dehydrogenase enzyme present in the viable cells to form orange colored formazan crystals. The absorbance of the orange colored formazan solution in each well of the 96 well plate was then spectrophotometrically measured at a wavelength of 490nm using a plate reader (POLARstar Optima, BMG LABTECH, Durham, NC). The percentage of cells surviving and the concentration at which 50% of the cells remain viable after 72h sample treatment (LD_{50}) were determined. Each sample was tested in triplicate and their mean values were taken to calculate the cell viability and LD_{50}. Cell viability (%) = $[(A_s - A_b)/A_v \times 100]$ where A_s = Absorbance of test sample well (well with sample+cells+media), A_b = Absorbance of blank control well (well with sample+media but no cells), A_v = Absorbance of vehicle control well (well with cells+DMSO of 0.5%).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Extraction of medicinal plants

The main aim of the preparative extraction method is the identification of the major or bioactive compound(s) from a specific plant. The concentrations (mg of dissolved solids per mL of solvent) of the extracts produced are shown in **Table 3**.

Table 3- Dissolved solid extracts produced

Plant	**Organic extract**	**Aqueous extract**
Goldenseal	219mg/mL	38.7mg/mL
Turmeric	568.8mg/mL	44mg/mL
Ginger	13.4mg/mL	39.8mg/mL
Lesser galangal	29.9mg/mL	25.4mg/mL

These weights were obtained by transferring 1mL of extracts into eppendorf tubes and then drying them using a speedvac evaporator. All these extracts were transferred to a suitable glass container and then stored in a refrigerator (4°C) for subsequent analysis.

3.2 Major components identified from plant extracts using HPLC-ESI-MS

The organic and aqueous extracts of all the four medicinal plants were analyzed using reversed phase high performance liquid chromatography on a C18 column and electrospray ionization mass spectrometry method. Significant peaks were observed in only organic extracts and were absent in aqueous extracts.

3.2.1 Analysis of *Curcuma longa* extract

The base peak chromatogram of *C. longa* organic extract shows a significant peak at a retention time of 21.45 min (**Figure 5**). **Figure 6** shows a series of selected ion chromatograms for different concentrations of turmeric (*Curcuma longa*) organic extract carried out in the negative ion mode. Since the phenolic group in the structure of the major component curcumin can be easily deprotonated, the negative ion mode scanning was selected. In selected ion chromatograms, ions with selected m/z values (those that correspond to the masses of particular components of the samples) are plotted as a function of time. This type of monitoring increases the selectivity for individual analytes and improves the detection limit by decreasing the background noise. In these chromatograms, an m/z value of 367 was selected since the molecular mass of neutral curcumin was 368. The major molecular ion present has an m/z ratio of 367 (deprotonated curcumin) and has been identified as **curcumin** with reference to literature[30]. In addition, the retention time was found to be similar to that of the standard curcumin which is shown in **Table 4**.

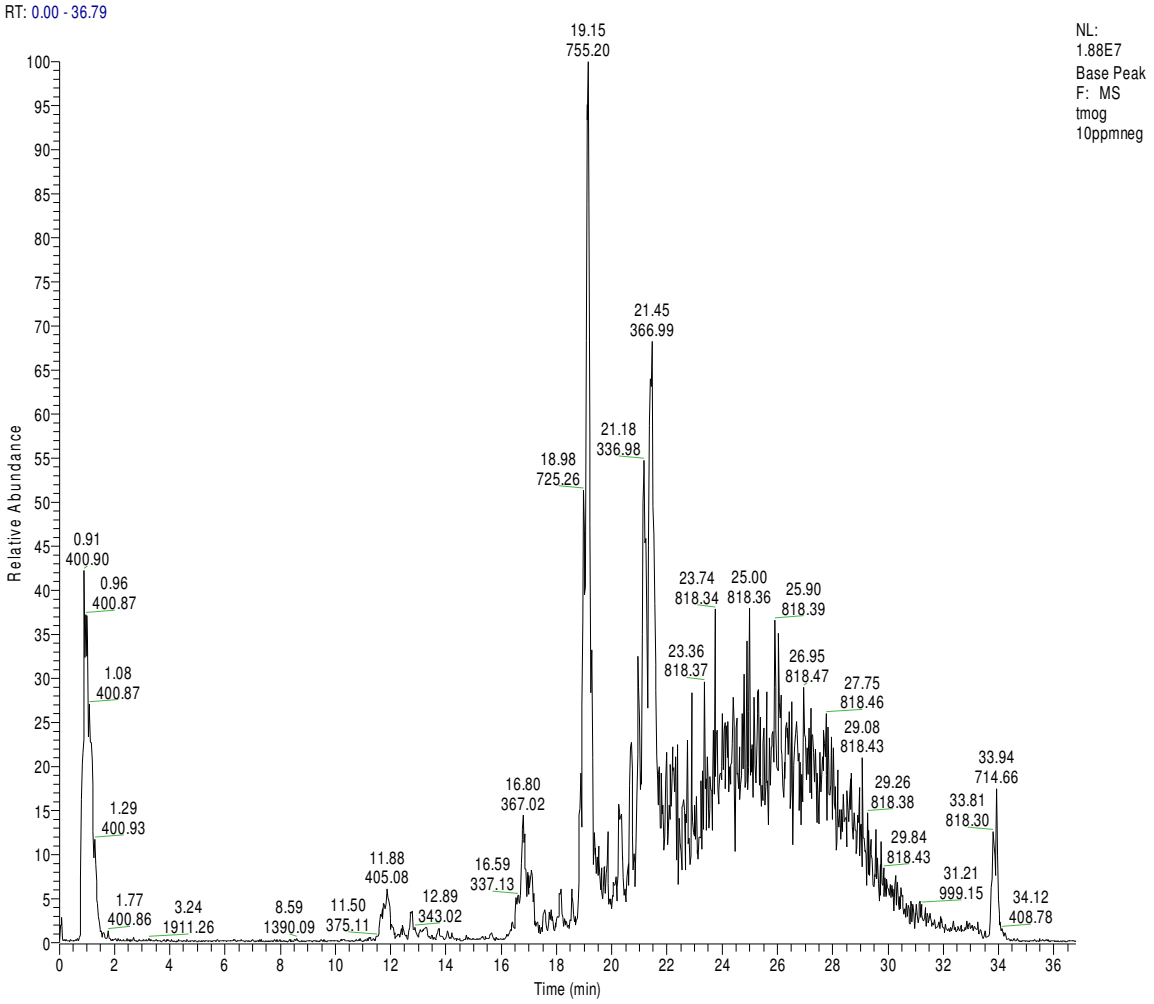


Fig 5 – *Base peak chromatogram of turmeric organic extract obtained using HPLC-ESI-MS*

Table 4 – Comparison of retention times of curcumin for both standard and original extract

Curcumin	**Avg. retention time**	**Std. Dev (N= 3)**
Standard	20.63 min	0.049
Extract	20.55 min	0.055

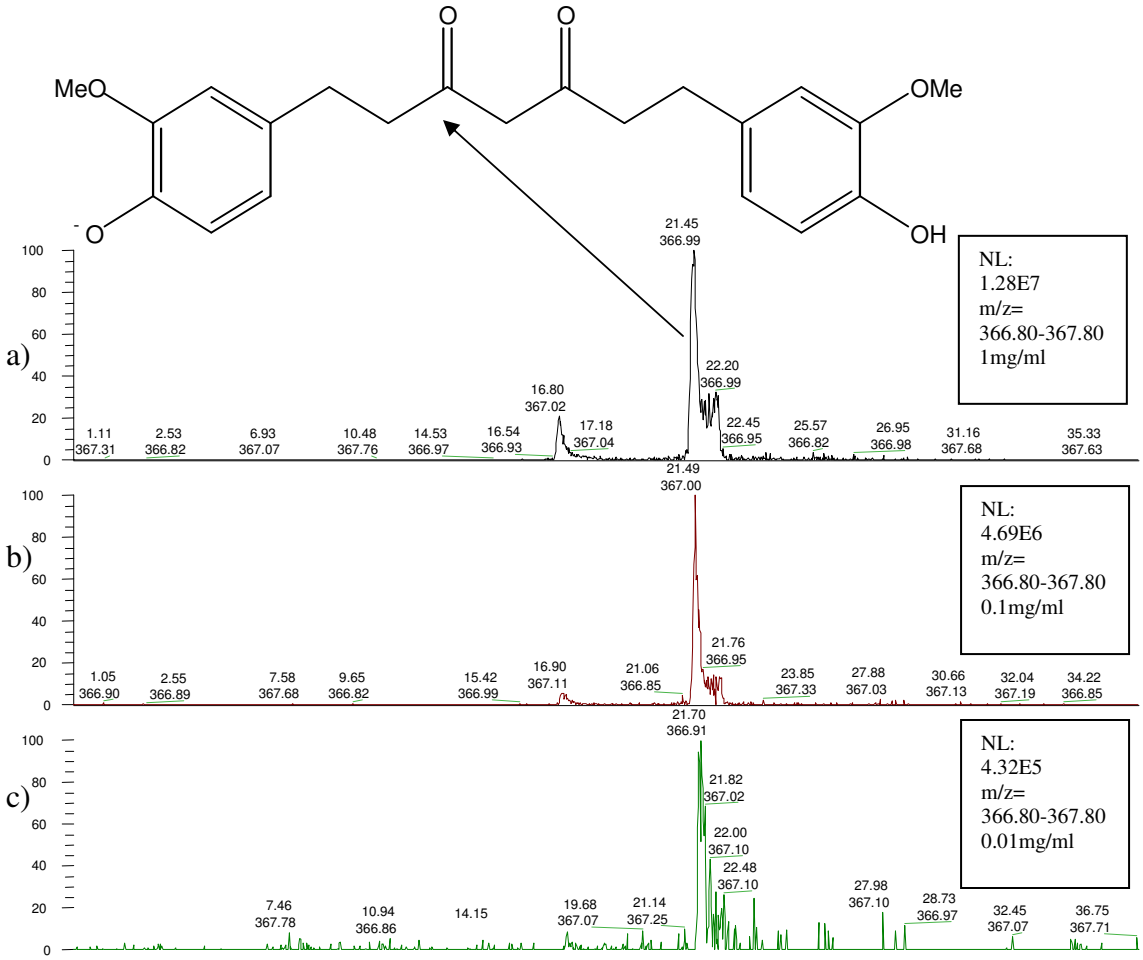


Fig 6 – *Selected ion chromatogram of turmeric organic extract obtained using HPLC-ESI-MS. Three concentrations were analyzed, a) 1mg/mL b) 0.1mg/mL and c) 0.01mg/mL (expressed as mg dissolved solids/mL solvent). The m/z value of 337 was plotted, which corresponds to mass of deprotonated curcumin (see structure in chromatogram a.)*

It can be concluded from the above chromatogram that the peak size increases with the increasing concentration of the extract (i.e. 0.01 mg/mL to 1 mg/mL), as would be expected.

And also the presence of the compound curcumin was confirmed by comparing the MS-MS spectrum of standard curcumin with that of the peak at a retention time of 21.45 min (**Figure 7**).

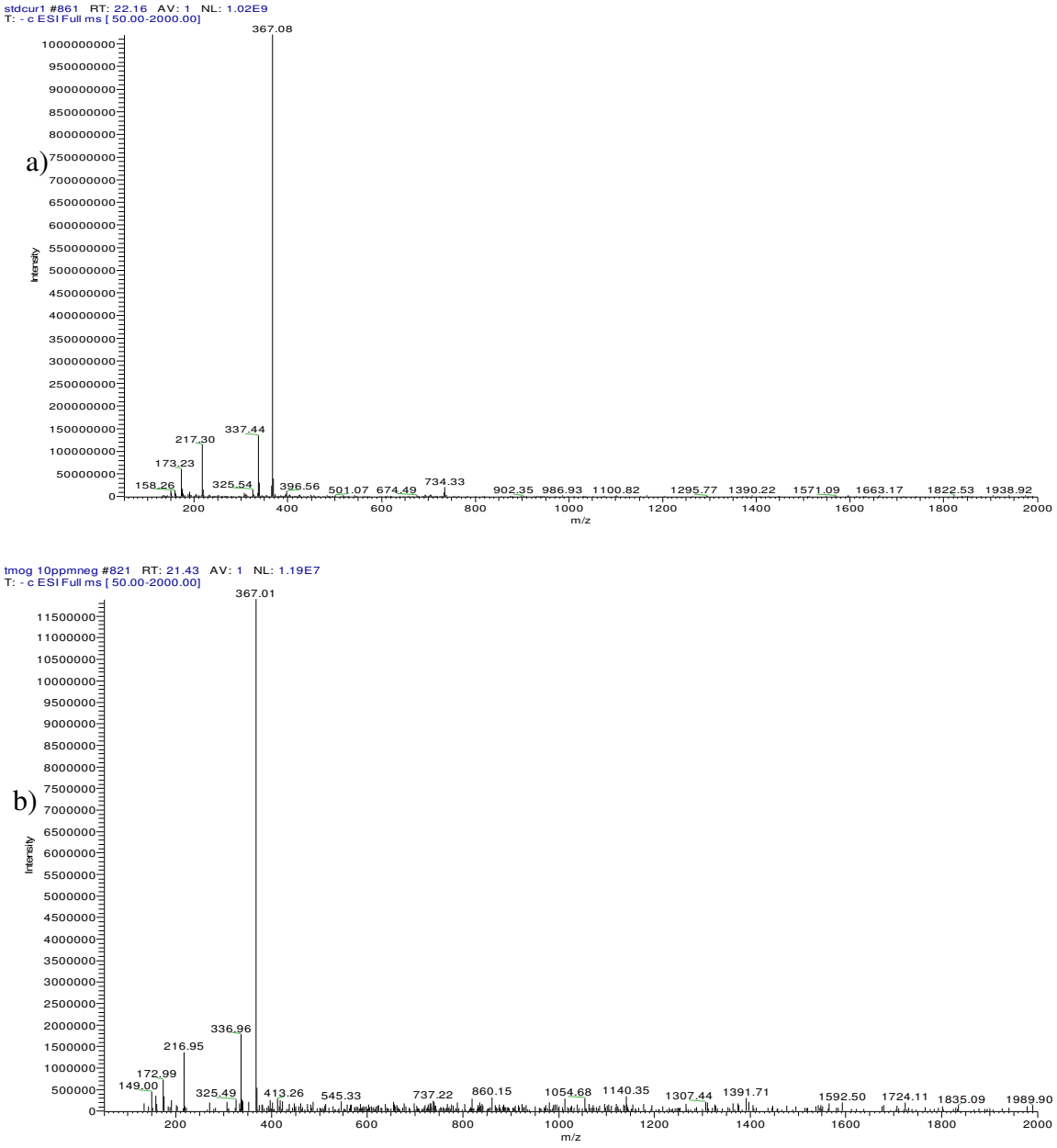


Fig 7 - *Full scan mass spectra for (a) standard curcumin (molecular weight of 368) and (b) the peak obtained at a retention time of 21.45 min for turmeric organic extract*

3.2.2 Analysis of *Hydrastis canadensis* extract

For the analysis of goldenseal (*Hydrastis canadensis*) organic extract, carried out in the positive ion mode scanning, the major molecular ion observed has a m/z ratio of 336 which was been identified as **berberine** with reference to literature[51]. The berberine molecule, which has a quaternary nitrogen atom, exists as a cation. Hence the positive ion mode was used. A series of selected ion chromatograms for three different concentrations of goldenseal are shown in **Figure 8**. Note that without comparison to a standard, identification of berberine is only tentative.

3.2.3 Analysis of *Alpinia officinarum* extract

The major molecular ions present in the selected ion chromatograms for lesser galangal (*Alpinia officinarum)* organic extract (**Figures 9, 10 and 11***)* have m/z ratios of 285, 269 and 301. These ions were tentatively identified as **kaempferol, galangin,** and **quercetin,** respectively, with reference to literature[52]. This analysis was performed in the negative ion mode.

Zerumbone[53], the major component of ginger (*Zingiber officinalis*), could not be detected as a single molecular ion peak using HPLC-ESI-MS because it cannot easily be protonated or deprotonated. It can be analyzed using GCMS.

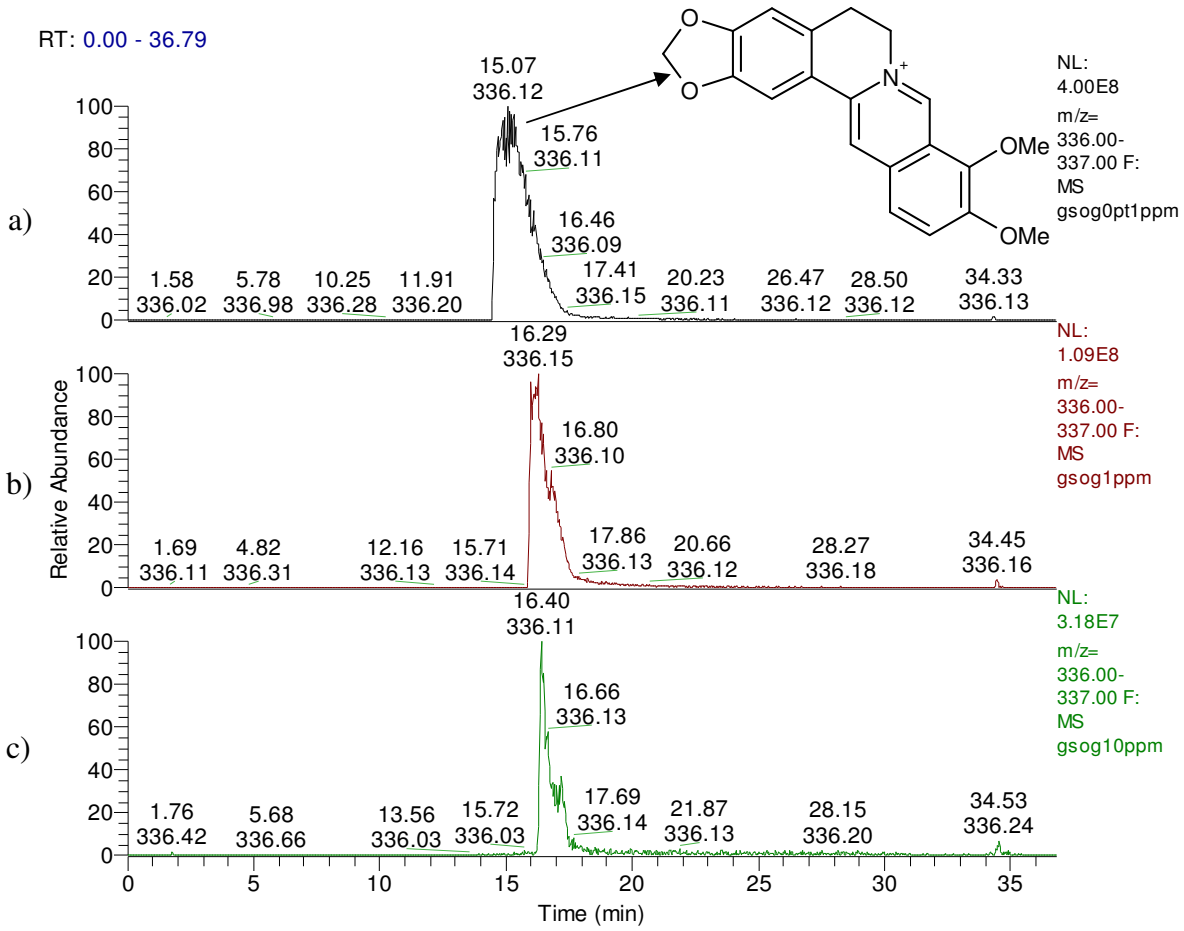


Fig 8 – *Selected ion chromatogram of m/z 336 from goldenseal organic extract obtained using HPLC-ESI-MS for three different concentrations a) 1mg/mL b) 0.1mg/mL c) 0.01mg/mL. The m/z value of 336 correlates with that of berberine (structure shown in chromatogram a.)*

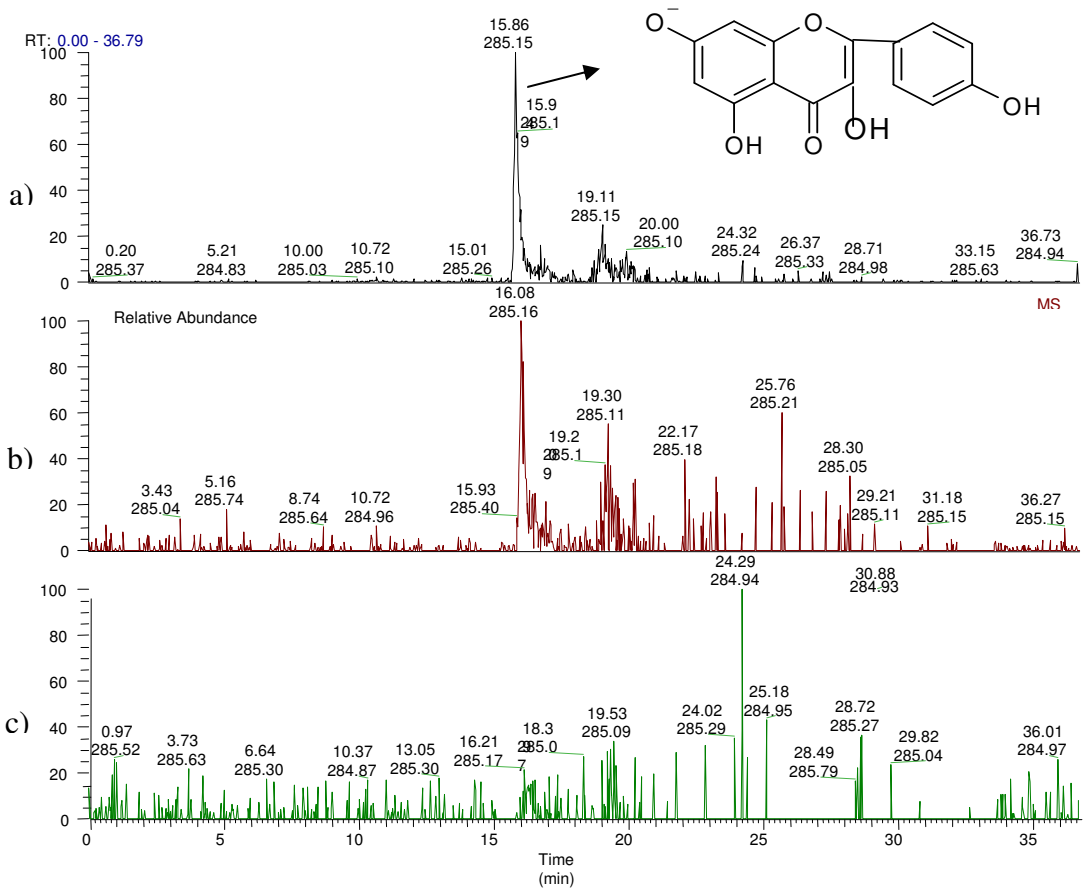


Fig 9 – *Selected ion chromatogram of* A. officinarum *organic extract for m/z value of 285 obtained using HPLC-ESI-MS. a) 1mg/mL b) 0.1mg/mL c) 0.01mg/mL. The m/z value of 285 corresponds to the mass of deprotonated kaempferol*

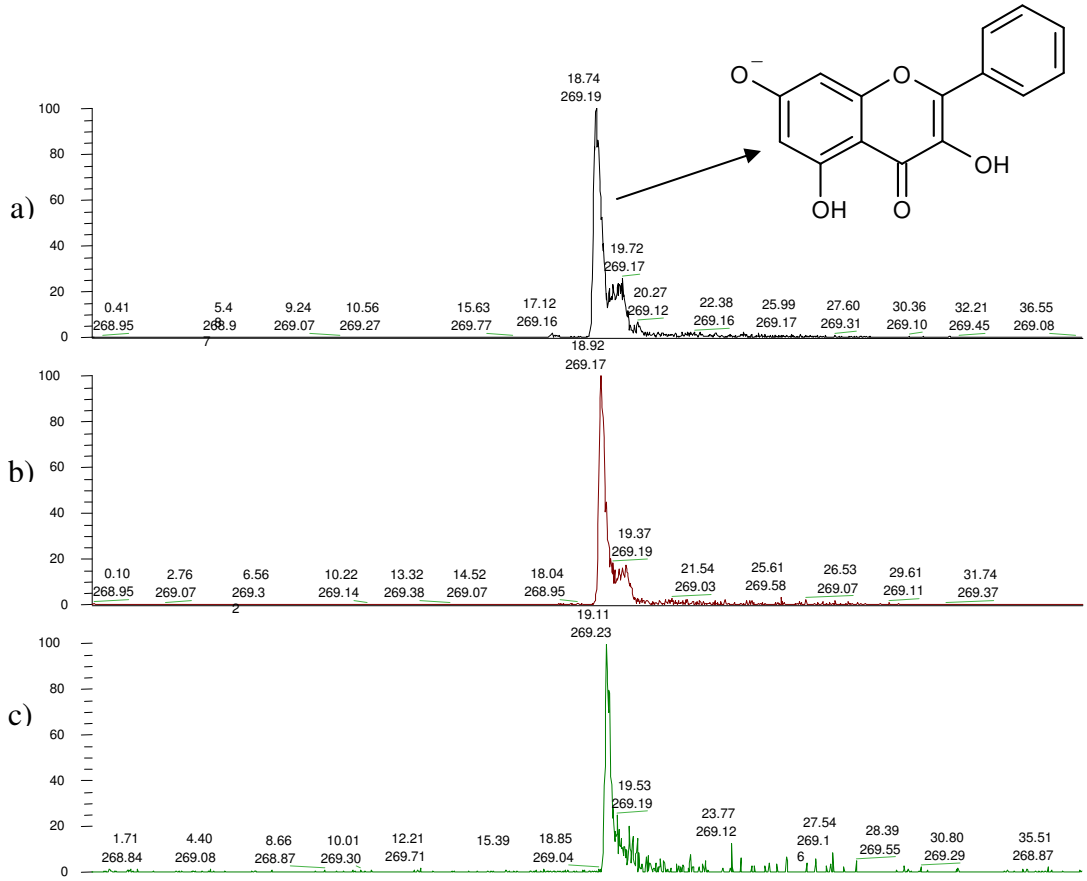


Fig 10 – *Selected ion chromatogram of* A. officinarum *organic extract for m/z value of 269 obtained using HPLC-ESI-MS. a) 1mg/mL b) 0.1mg/mL c) 0.01mg/mL. The m/z value of 269 corresponds to the mass of deprotonated galangin*

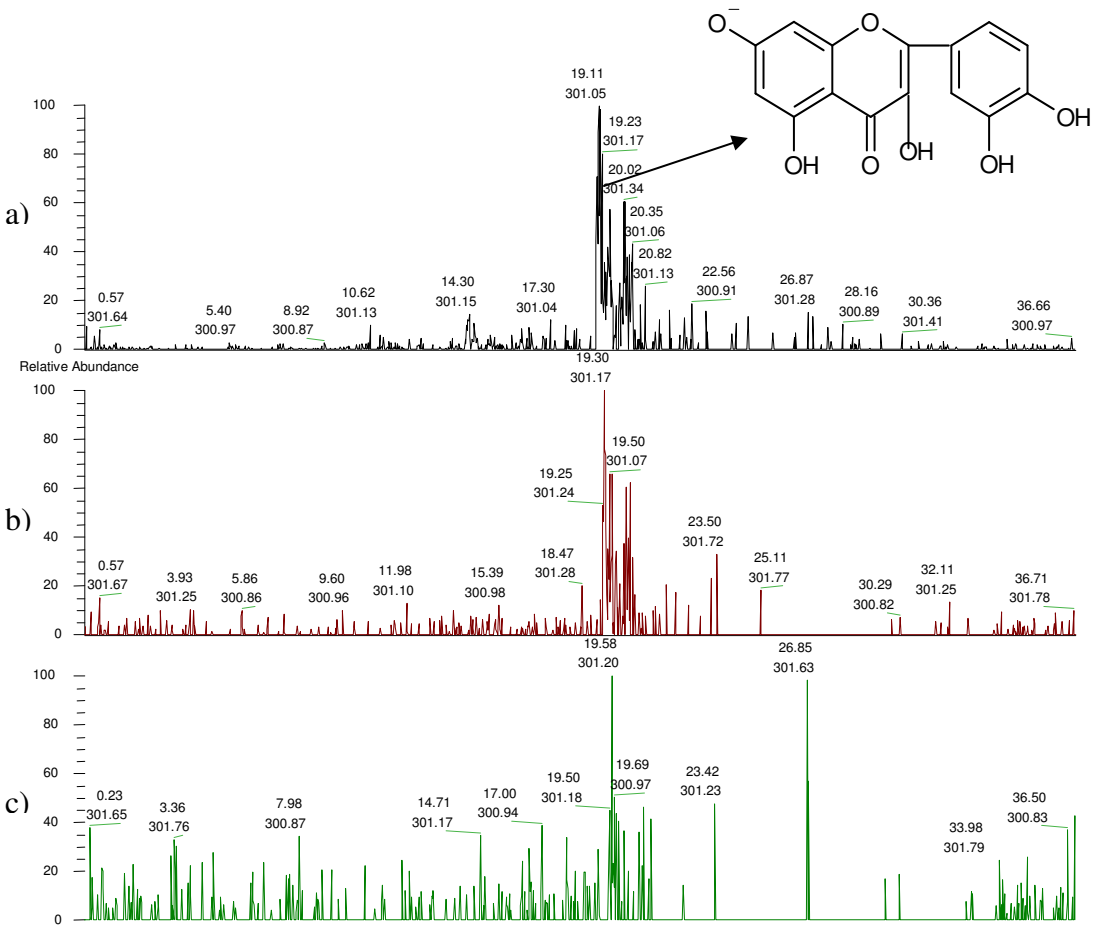


Fig 11 – *Selected ion chromatogram of* A. officinarum *organic extract for m/z value of 301 obtained using HPLC-ESI-MS. a) 1mg/mL b) 0.1mg/mL c) 0.01mg/mL. The m/z value of 301 corresponds to the mass of deprotonated quercetin*

3.3 Anti-proliferative activity of plant extracts on *Dictyostelium* cells

Organic and aqueous extracts of all the four medicinal plants were tested for anti-proliferative activity using *Dictyostelium* cells. All of the aqueous extracts and the goldenseal organic extract showed no inhibition of cell growth. Organic extracts of *Curcuma longa*, *Zingiber officinalis*, and *Alpinia officinarum* demonstrated significant concentration dependent anti-proliferative activity as shown in **Tables 5, 6, and 7** and **Figures 12, 13, and 14,** respectively. From the data obtained, it was found that the organic extracts of *C. longa*, *A. officinarum*, and *Z. officinalis* have shown similar anti-proliferative activity profiles with *Dictyostelium* cells.

Table 5 – *Dictyostelium* cell growth in the presence of different concentrations of *C. longa* organic extract

Days	CELL NUMBER			
	Turmeric 17 µg/mL	**Turmeric 8.5 µg/mL**	**Turmeric 4.2 µg/mL**	**DMSO Control**
0	5	5	5	5
1	6	7	9	7
4	3	19	95	325
5	17	7	210	400

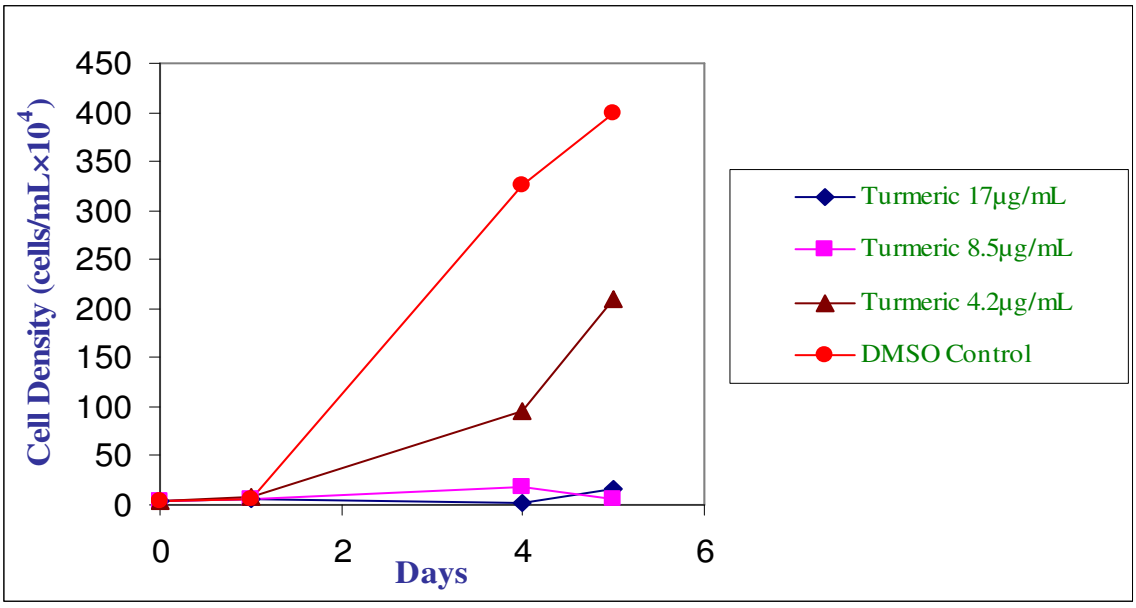


Fig 12 – *Effect of* C. longa *organic extract on* Dictyostelium *cell growth*

Table 6 – Effect of different concentrations of *A. officinarum* organic extract on *Dictyostelium* cell count

Days	CELL NUMBER			
	***Alpinia off.* 17 µg/mL**	***Alpinia off.*8.5 µg/mL**	***Alpinia off.*4.2 µg/mL**	**DMSO Control**
0	5	5	5	5
1	4	6	11	18
2	4	5	22	23
3	9	7	24	52
4	3	8	110	240

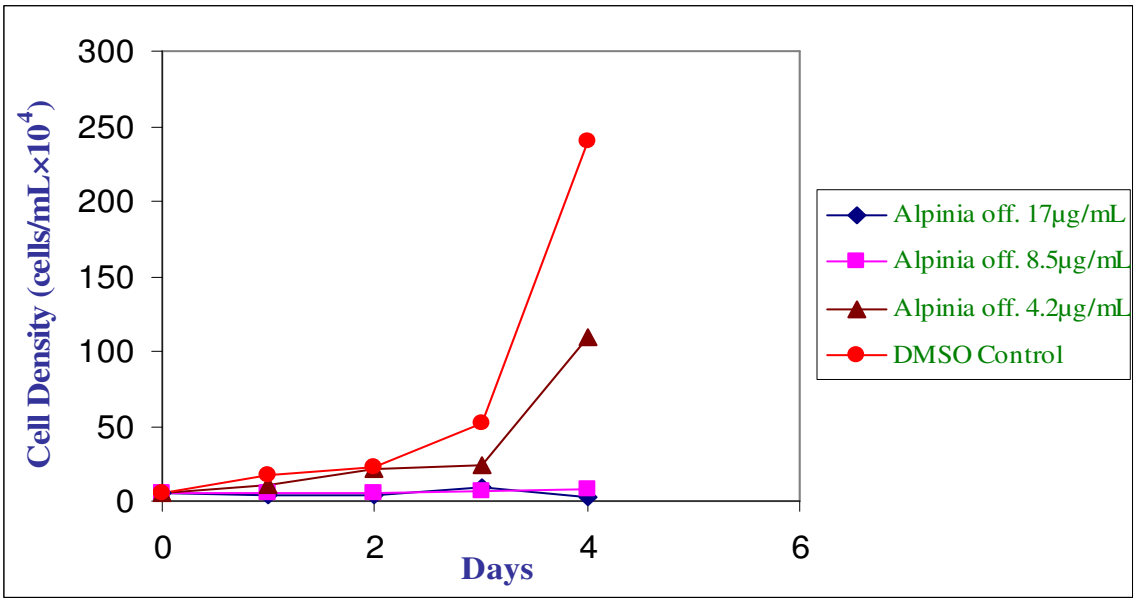


Fig 13- *Effect of* A. officinarum *organic extract on* Dictyostelium *cell growth*

Table 7 – *Dictyostelium* cell growth in the presence of *Z. officinalis* organic extract

Days	CELL NUMBER			
	Ginger 17 µg/mL	**Ginger 8.5 µg/mL**	**Ginger 4.2 µg/mL**	**DMSO Control**
0	5	5	5	5
1	3	1	16	18
2	1	24	32	23
3	3	38	53	52
4	3	100	220	240

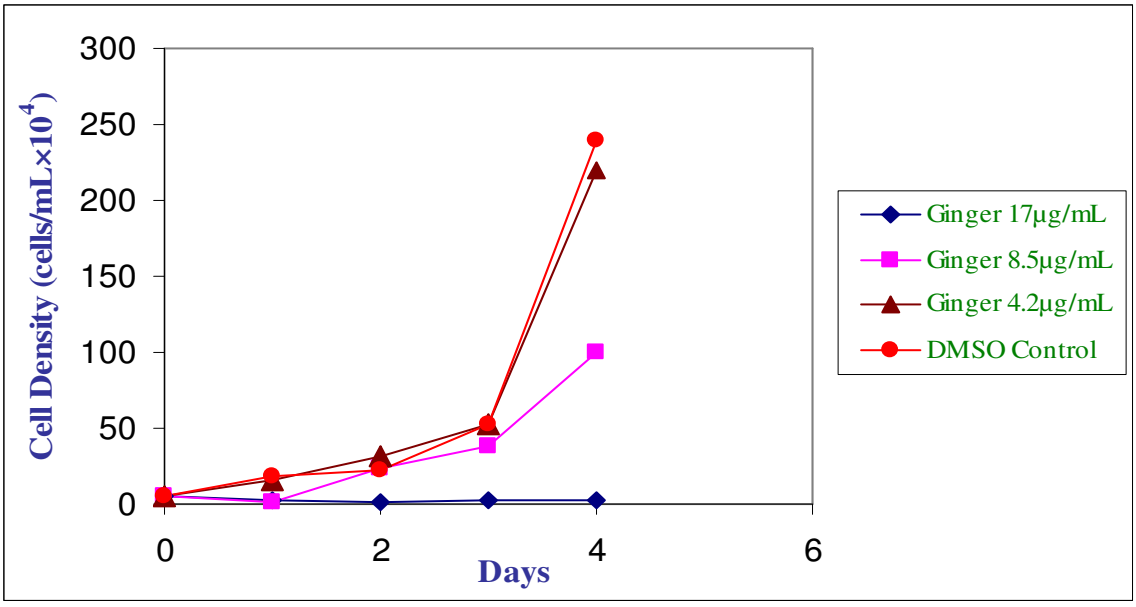


Fig 14 – *Effect of* Z. officinalis *organic extract on* Dictyostelium *cell growth*

3.4 Quantitation of curcumin in turmeric fractions

Given the promising results and therapeutic significance of turmeric (*C. longa*), this plant was chosen as the subject of further study. The *C. longa* extract was fractionated over silica gel using a binary solvent mixture of hexane and diethyl ether (see Methods Section 2.4) and the resulting fractions were analyzed for concentration of curcumin. From the data obtained, it was evident that all the turmeric fractions contained curcumin (ion with an m/z ratio of 367) except fraction 1. The concentration of this component in each fraction was determined from the calibration curve shown in **Figure 15**. This calibration curve was plotted as log of concentration versus the log of peak area of the selected ion chromatogram for the ion with m/z 367 and retention time 20.60 min.

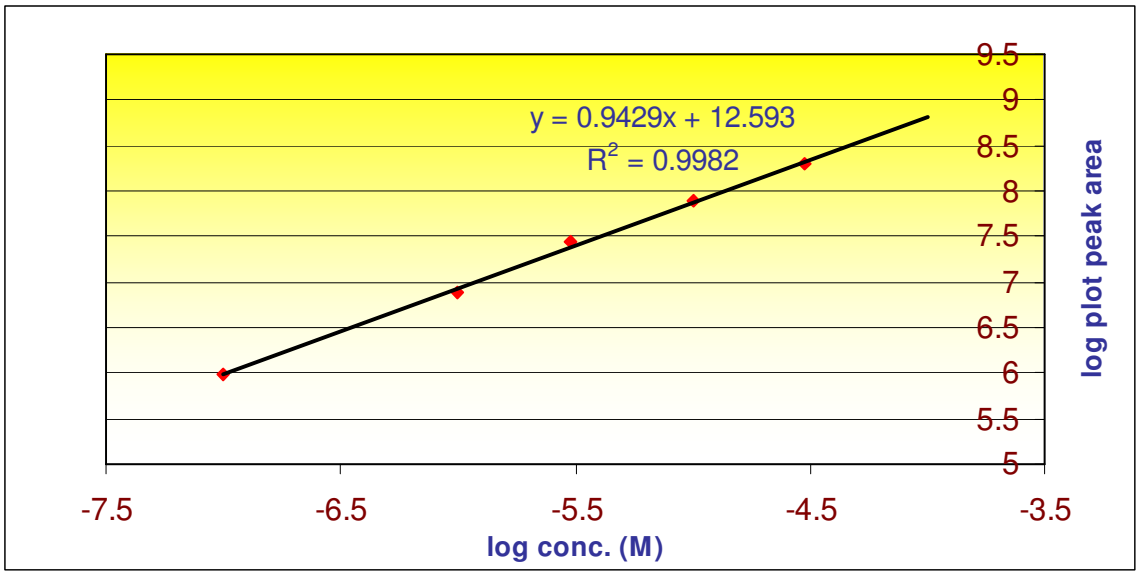


Fig 15 - *Calibration curve for curcumin*

From the calibration curve, the concentrations of the turmeric fractions collected were obtained. Fraction 8 has the highest concentration of curcumin, whereas fraction 6 has the least curcumin concentration (**Figure 16**). **Table 8** shows the concentrations of curcumin in the turmeric fractions collected. Standard deviations were calculated from statistical analysis of the calibration curve data.

Table 8 – Concentrations of curcumin from different fractions of *C. longa* organic extract including the original extract (Wt% is mg curcumin per mg dissolved solids in extract × 100)

Fraction no.	**Conc. (mg/mg) +/- Std. Dev.**	**Wt% +/- Std. Dev**
1 (10% Diethyl Ether)	_____	_____
2 (20% Diethyl Ether)	0.076 ± 0.021	7.6 ± 2.1
3 (30% Diethyl Ether)	0.114 ± 0.031	11.4 ± 3.1
4 (40% Diethyl Ether)	0.034 ± 0.009	3.4 ± 0.9
5 (50% Diethyl Ether)	0.049 ± 0.014	5.0 ± 1.4
6 (60% Diethyl Ether)	0.021 ± 0.006	2.1 ± 0.6
7 (70% Diethyl Ether)	0.200 ± 0.055	20.0 ± 5.5
8 (85% Diethyl Ether)	0.418 ± 0.117	42.0 ± 12.0
9 (100% Diethyl Ether)	0.151 ± 0.041	15.1 ± 4.1
Original extract	0.118 ± 0.032	12.0 ± 3.2

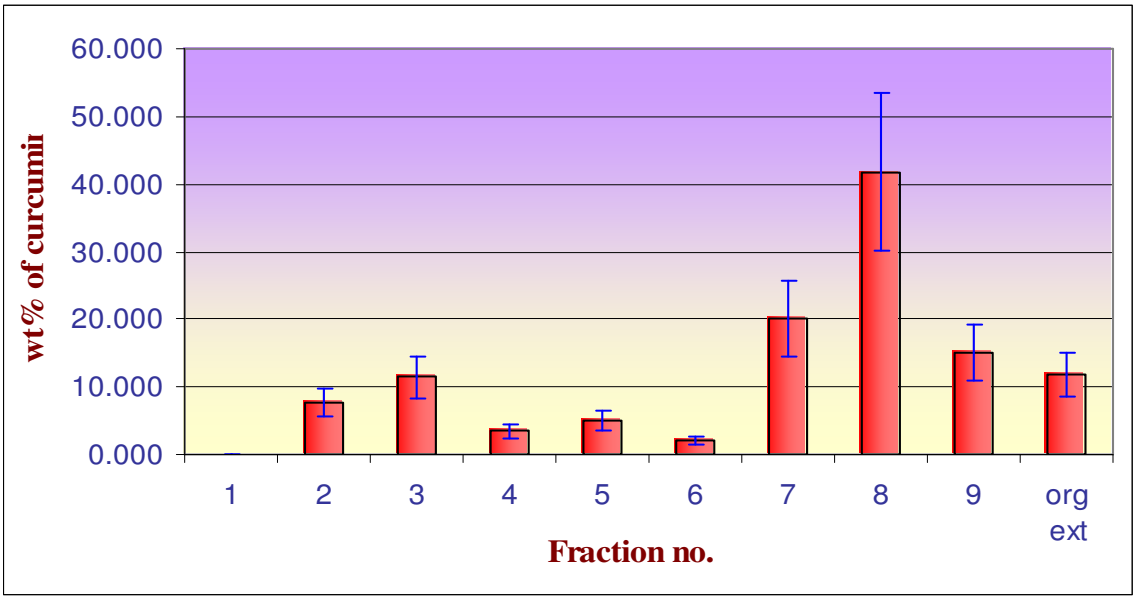


Fig 16 - *Concentrations of curcumin in the turmeric extract fractions determined by HPLC-ESI-MS*

3.5 Other curcuminoids identified in the turmeric fractions

After determining the curcumin concentrations in the turmeric fractions, the next step in this phytochemical screening would be to identify and quantify the other two curcuminoids present. Demethoxycurcumin (MW - 338) and bisdemethoxycurcumin (MW - 308) were identified in all the turmeric fractions except in fraction 1. The relative amounts of these curcuminoids in the various fractions were then determined based on the peak areas obtained for the selected ion chromatograms for the ions with m/z values of 337 and 307 (**Figure 17 and Figure 18**). Absolute quantification of these compounds was not possible given that standards were not commercially available. As with curcumin, fraction 8 has the highest concentration of the curcuminoids.

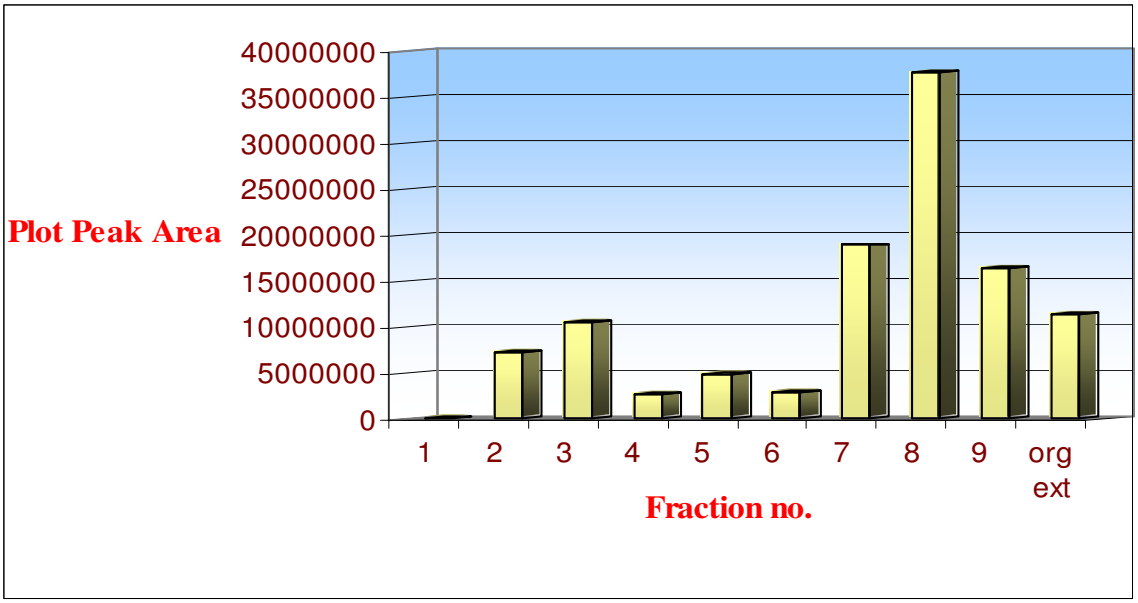


Fig 17 – *Relative amounts of demethoxycurcumin present in the turmeric fractions*

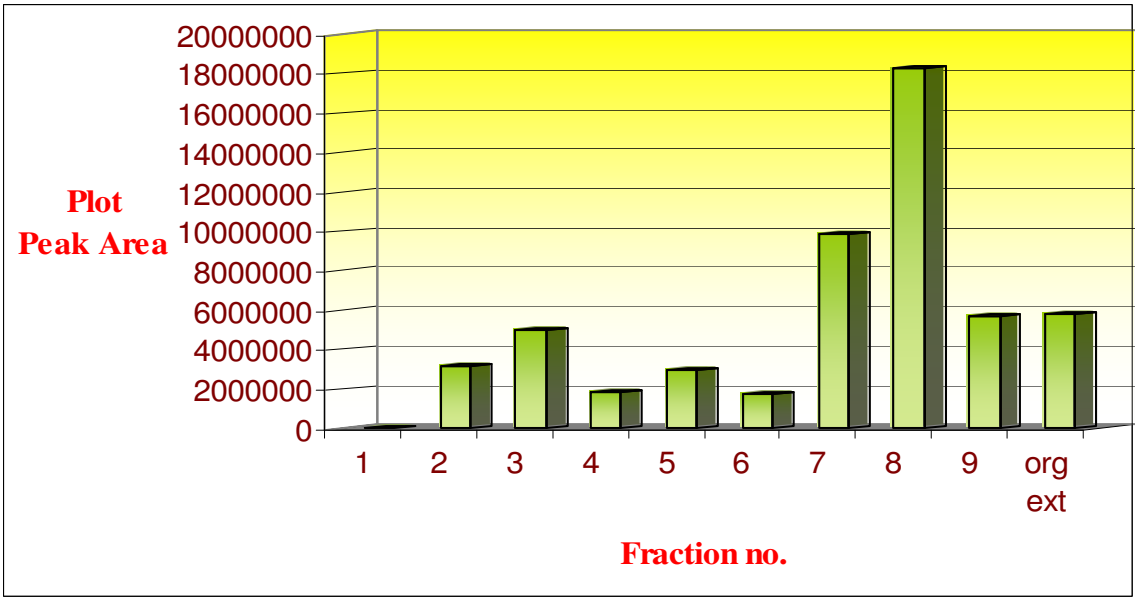


Fig 18 – *Relative amounts of bisdemethoxycurcumin present in the turmeric fractions*

3.6 Identification and quantitation of ar-tumerone in the turmeric fractions

All the turmeric fractions including the original extract were analyzed for the presence of volatile oil constituents using positive mode HPLC-ESI-MS method. Significant peaks were observed only in fraction 1 and original extract samples. Volatile oil components like ar-tumerone, curlone and α-tumerone were all identified with reference to literature[35] (**Figure 19**).

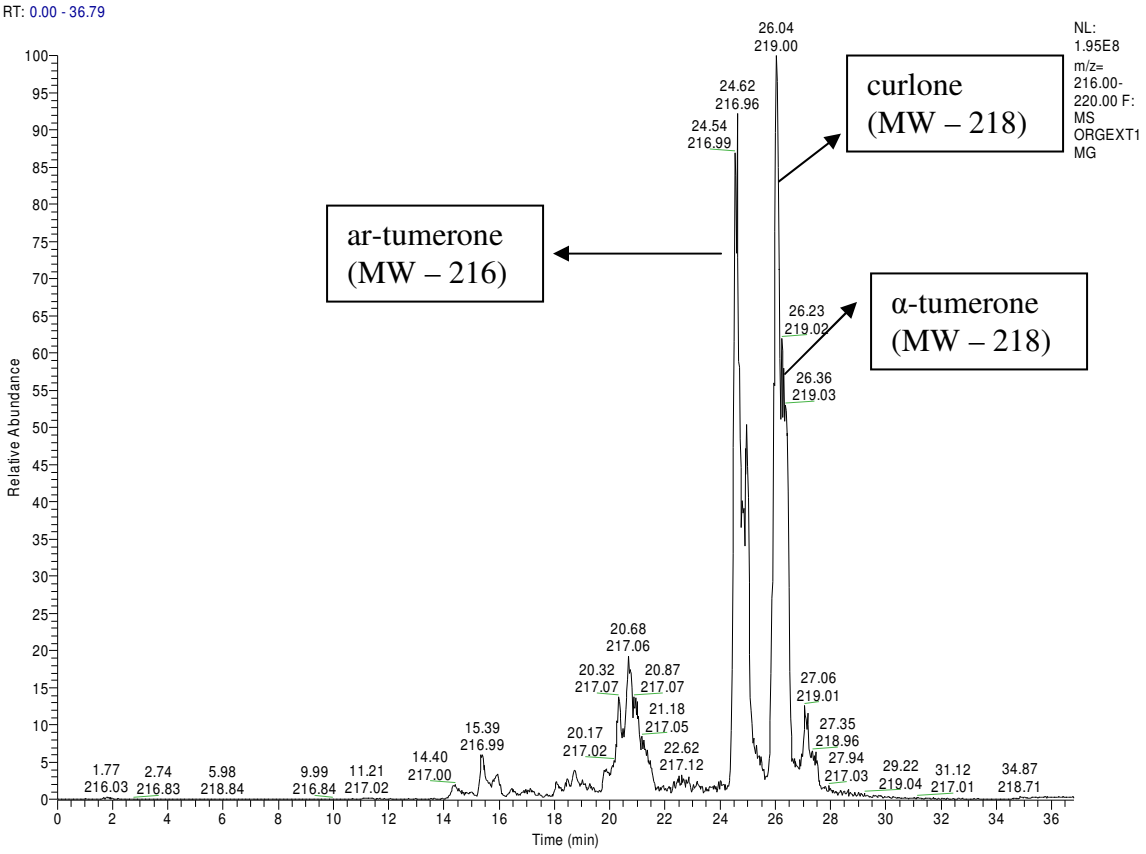


Fig 19 – *Total ion chromatogram (mass range 216-220) of the turmeric original extract obtained using positive mode HPLC-ESI-MS. Three volatile oil components of m/z values of 217 and 219 were been identified*

The peak with an m/z value of 219 corresponds to the presence of two volatile oil constituents curlone and α-tumerone. Since they have the same m/z values they co-elute together with curlone being the first and α-tumerone the latter[35]. Since ar-tumerone was the compound of interest, selected ion chromatogram of this compound (MW-217) was plotted. **Figures 20 and 21** represent the selected ion chromatograms of m/z value of 217 for three different concentrations of fraction 1 and original extract samples. The peak with an m/z value of 217 represents the protonated form of neutral ar-tumerone[35]. The presence of this compound ar-tumerone was also confirmed by comparing the retention time with that of the standard ar-tumerone (**Table 9**). The tentative structure for protonated ar-tumerone compound is shown in Figures 20 and 21.

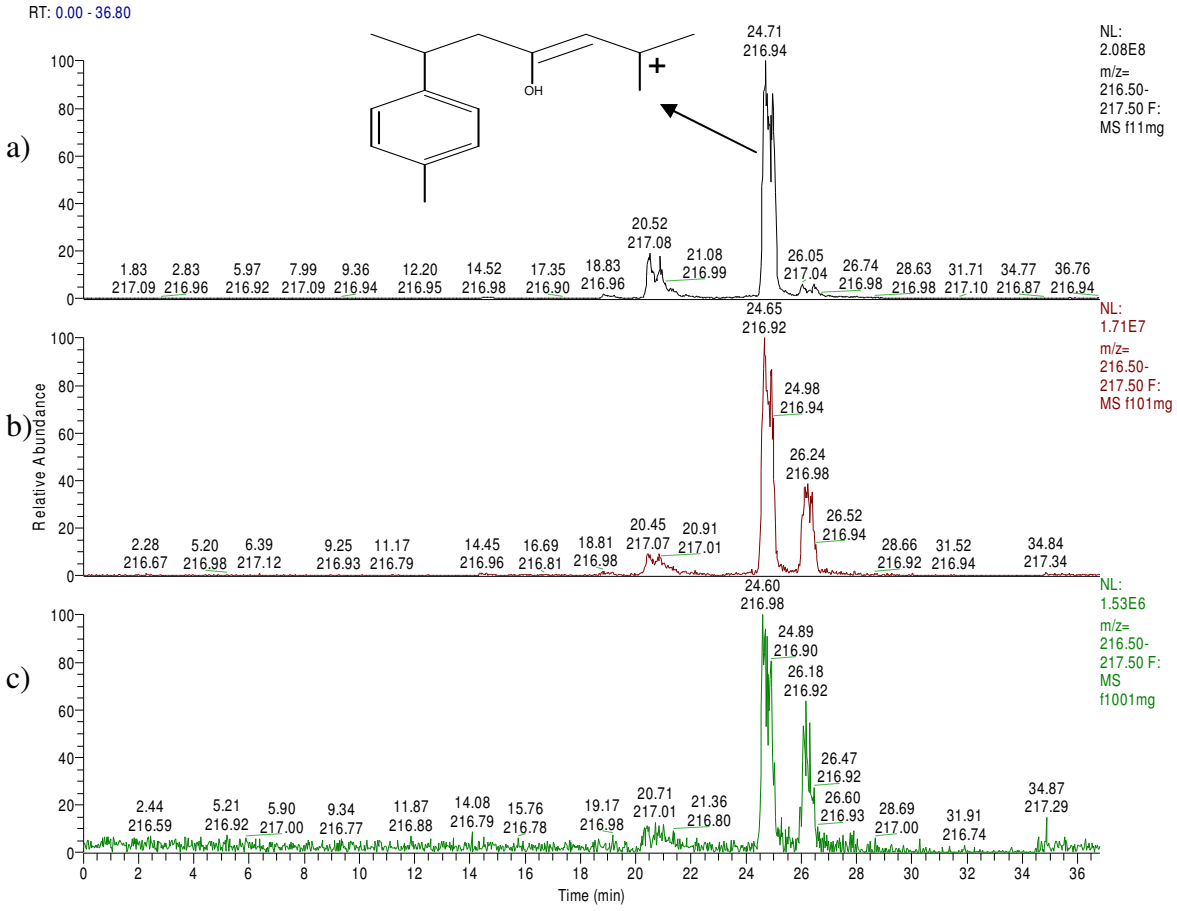


Fig 20 – *Selected ion chromatogram of m/z value of 217 obtained using positive mode HPLC-ESI-MS for three different concentrations of turmeric fraction 1. a) 1mg/mL b) 0.1mg/mL c) 0.01mg/mL. The m/z value of 217 corresponds to the mass of protonated form of ar-tumerone*

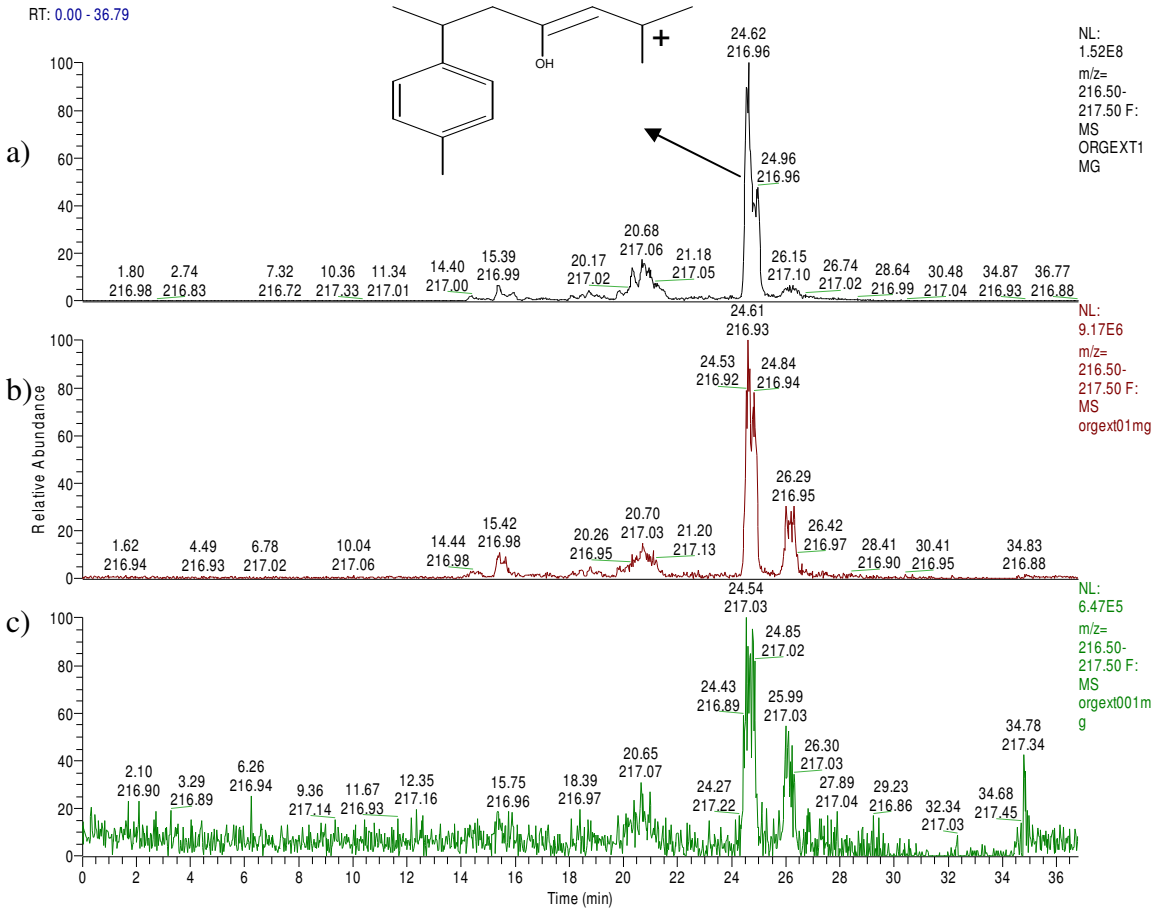


Fig 21 – *Selected ion chromatogram of turmeric original extract obtained for an m/z value of 217 using positive mode HPLC-ESI-MS. Three different concentrations of a) 1mg/mL b) 0.1mg/mL c) 0.01mg/mL were analyzed. The m/z value of 217 corresponds to the mass of protonated ar-tumerone*

Table 9 – Comparison of retention times for standard ar-tumerone and the ion with m/z value of 217 in the turmeric original extract

Ar-tumerone	**Avg. retention time**	**Std. dev (N= 3)**
Standard	22.86 min	0.046
Turmeric extract	22.90 min	0.098

Figure 22 shows the MS-MS spectra of standard ar-tumerone and the peak obtained at a retention time of 24.62 min for turmeric original extract.

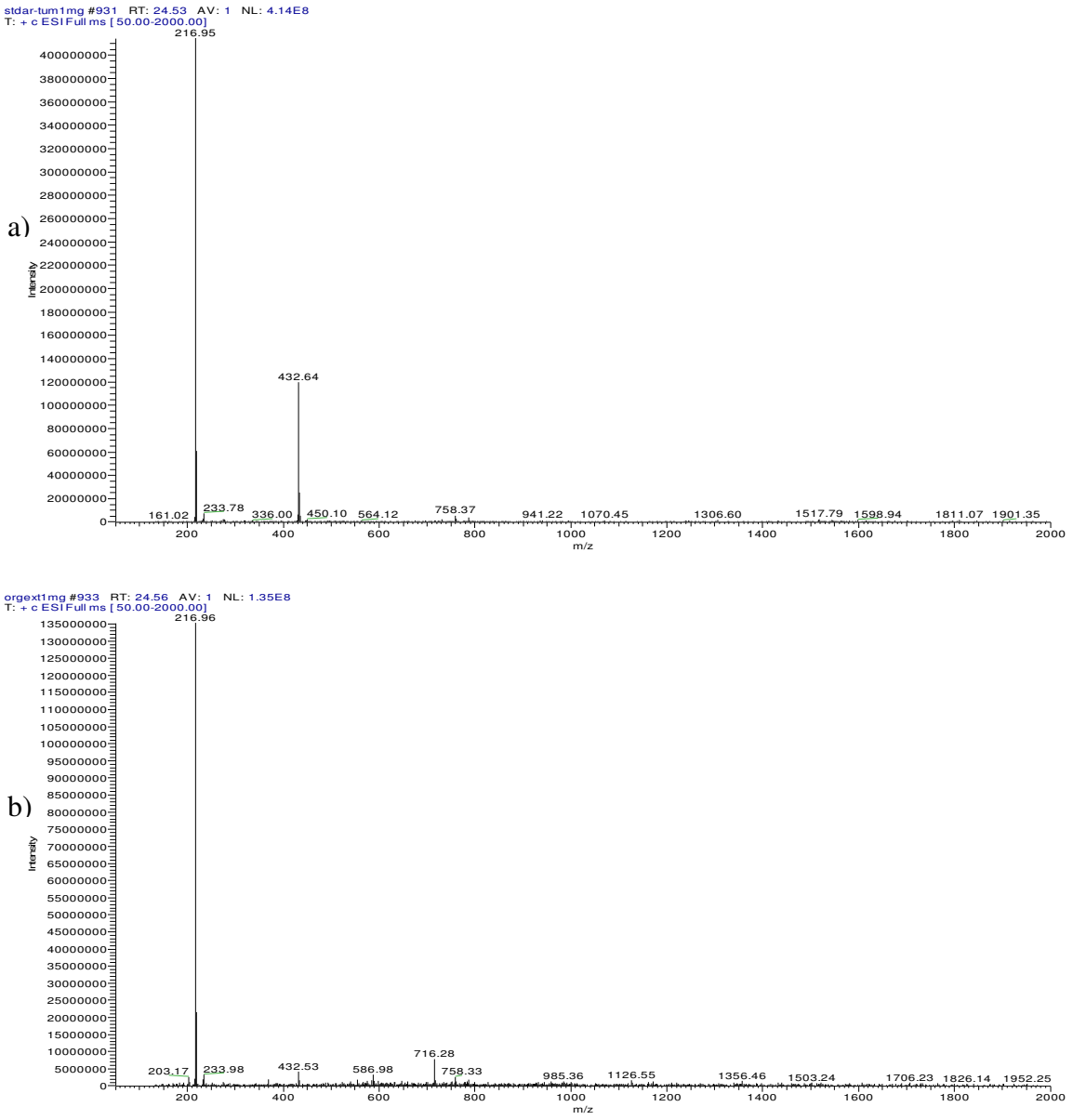


Fig 22 – *Full scan mass spectra of (a) standard ar-tumerone (molecular weight of 216) and (b) the peak at a retention time of 24.62 min for turmeric original extract*

Further, the concentration of ar-tumerone in fraction 1 and original extract was determined from the calibration curve of standard ar-tumerone as shown in **Figure 23**.

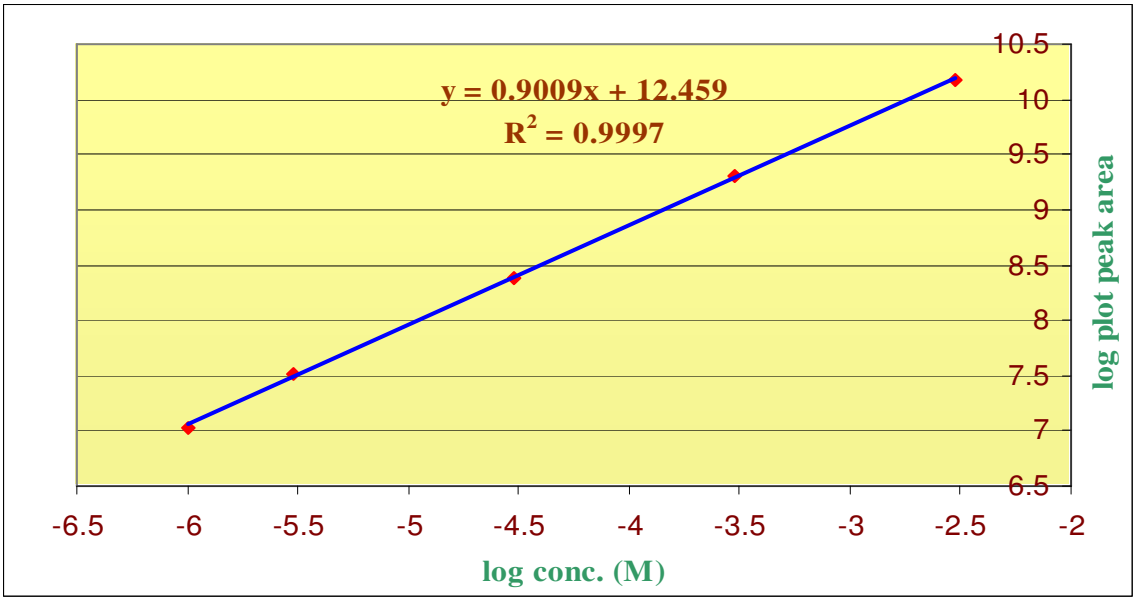


Fig 23 – *Calibration curve for ar-tumerone obtained by plotting log of concentration vs. log of peak area of the selected ion chromatogram for the ion of m/z 217*

Based on the calibration curve data, the concentrations of ar-tumerone in fraction 1 and turmeric original extract were calculated as shown in **Table 10** and **Figure 24**.

Table 10 – Concentrations of ar-tumerone present in fraction 1 and turmeric original extract (wt% is mg of ar-tumerone per mg of dissolved solid extract × 100)

Sample	Conc. (mg/mg) +/- Std. Dev.	Wt% +/- Std. Dev
Fraction 1	0.078 ± 0.004	7.8 ± 0.4
Turmeric original extract	0.028 ± 0.001	2.8 ± 0.1

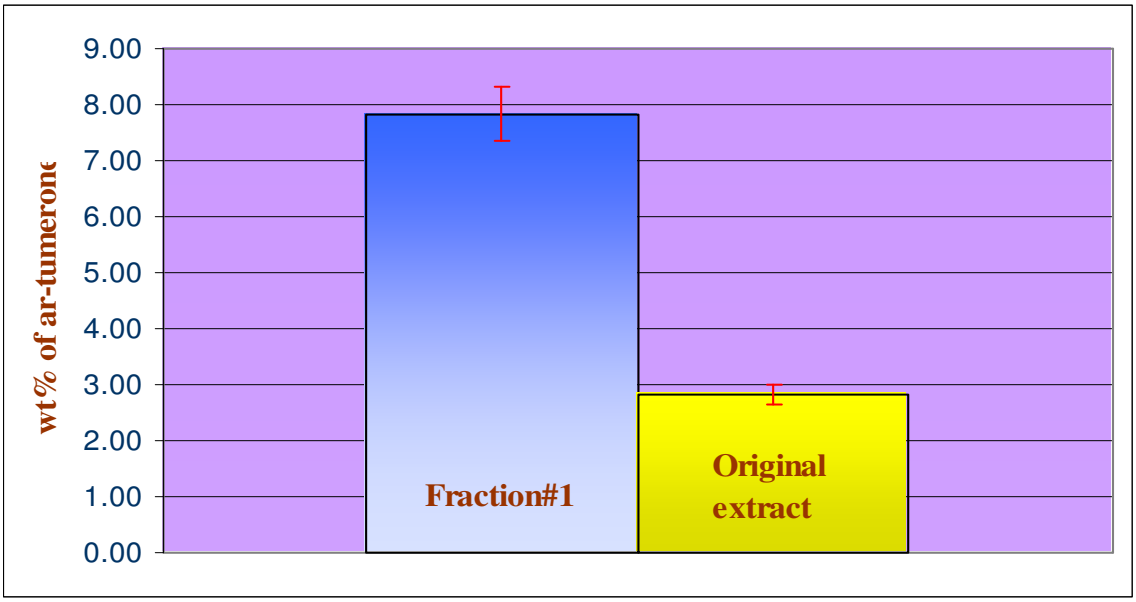


Fig 24 – *Concentrations of ar-tumerone in turmeric fraction 1 and original extract determined by positive mode HPLC-ESI-MS*

3.7 XTT assay results

In the present study, the anti-proliferative activity of all the turmeric fractions including the original extract was evaluated using MCF-7 human breast cancer cells. This was accomplished by measuring the viability of cells using the XTT assay (see Methods Section 2.6).

Table 11 and **Figure 25** demonstrate the MCF-7 cell growth in the presence of different concentrations of standard curcumin for 72h duration. The relative absorbance at 490nm (% of vehicle control) was plotted against concentration of curcumin. Only viable cells metabolize the XTT reagent and produce orange colored formazan crystals which absorb at 490nm. Therefore, the absorbance measured is proportional to the total number of viable cells. Standard curcumin inhibited the MCF-7 cell proliferation in a dose dependent manner. The LD_{50} of curcumin was found to be 10µM.

Table 11 – MCF-7 cell growth in the presence of different concentrations of standard curcumin at exposure time of 72h

Concentration (µM)	Cell viability (%) ± SD
0	100 ± 5.3
1	74.7 ± 3.8
3	61.2 ± 4.3
10	51.9 ± 3.2
30	25.0 ± 8.3
100	22.3 ± 6.0

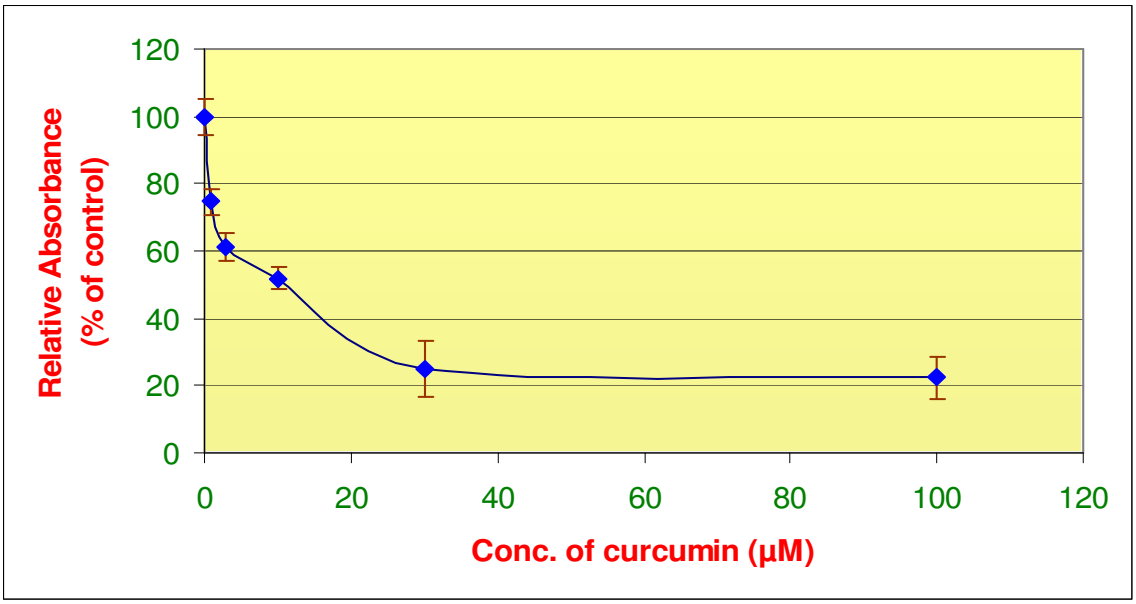


Fig 25 – *Effects of different concentrations of standard curcumin on MCF-7 cell proliferation. Relative Absorbance (% of vehicle control) vs. Concentration of curcumin was plotted to determine the LD_{50} of curcumin which was found to be 10µM*

Once the LD_{50} for curcumin against the MCF-7 cells had been determined, two different concentrations (3 and 30µM curcumin) of all the turmeric fractions were tested against MCF-7 cells using the XTT assay. The original extract and standard curcumin were also included in the assay. The approach used for testing these fractions was different than that typically employed for anti-proliferative screening of natural products. Typically, fractions were tested according to their dry weight, i.e. at concentrations of 2 and 20µg/mL. This approach is very useful for identifying the most active fraction from amongst a set of fractions (traditional bioassay guided fraction). However, it is not particularly useful for situations such as this one where all of the fractions contain a major active component (in this case curcumin) and the desired result is information about whether other minor constituents contribute also to the activity. To address the question of whether or not constituents other than curcumin contributed to the activity of the extract and fractions, a novel approach was employed. Relying on the quantitative analysis of curcumin in the isolated fractions (Table 8), each fraction (except fraction 1) was normalized to the same concentration of curcumin. Because fraction 1 did not contain curcumin, this fraction was tested at concentrations of 4 and 40µM of ar-tumerone, instead of as concentration of curcumin. By testing the fractions all with the same concentration of curcumin, it is possible to directly compare the results obtained for the fractions to those for standard curcumin alone at the same concentration. Any fractions that contain constituents that contribute (either additively or synergistically) to the activity of the fractions will show *greater activity* than curcumin alone at the same concentration. Any fractions that contain compounds that suppress the activity of the

curcumin will show less activity than curcumin alone at the same concentration. Finally, if the activity of the fractions is due solely to the presence of curcumin, these fractions will exhibit the same dose-response behavior as curcumin alone.

The results of the MCF-7 cell assay with the individual *C. longa* fractions are shown in shown in **Table 12** and **Figure 26.** Our data indicate that all the turmeric fractions and the complex turmeric extract containing curcumin exhibited similar potency (**Figure 27**). All the turmeric fractions (excluding fraction 1) including the original extract showed anti-proliferative activity similar to that of the standard curcumin. Fraction 1, which contained ar-tumerone and other volatile constituents but not curcumin, was found to be less effective (since the concentrations of this fraction used were 4 and 40μM of ar-tumerone). The complex turmeric extract which contains all the curcuminoids and ar-tumerone showed activity similar to that of the commercially available curcumin compound. All these results suggest that for this assay there is no major synergistic or additive activity observed for the various components present in the turmeric samples. Our results also suggest that the overall anti-proliferative activity of the turmeric extract is mainly due to the presence of the major component curcumin. Fraction 1 of the turmeric extract contained no curcumin but still had anti-proliferative activity; therefore, ar-tumerone and/or other volatile constituents may contribute somewhat to the anti-proliferative activity of *C. longa*. However, at the two concentrations of the complex extract tested, ar-tumerone was only present at concentrations of 9μM and 0.9μM. Therefore, its concentration was likely too low to result in significant activity.

Table 12 – Cell viability in the presence of different concentrations of turmeric fractions, original extract and standard curcumin at 72h exposure time ± SD

	% Cell viability ± SD	
	3µM	**30µM**
Fraction 1	91.9 ± 0.3	78.7 ± 4.9
Fraction 2	87.1 ± 5.1	54.7 ± 0.1
Fraction 3	84.6 ± 3.4	57.0 ± 6.5
Fraction 4	86.7 ± 3.9	56.5 ± 7.2
Fraction 5	83.8 ± 0.6	55.1 ± 0.4
Fraction 6	79.8 ± 3.1	56.5 ± 9.7
Fraction 7	85.0 ± 7.3	54.4 ± 0.8
Fraction 8	77.4 ± 4.1	43.5 ± 11.7
Fraction 9	86.4 ±3.8	55.8 ± 0.2
Original extract	85.6 ± 4.6	55.7 ± 1.5
Std curcumin	83.3 ± 9.2	55.6 ± 2.5

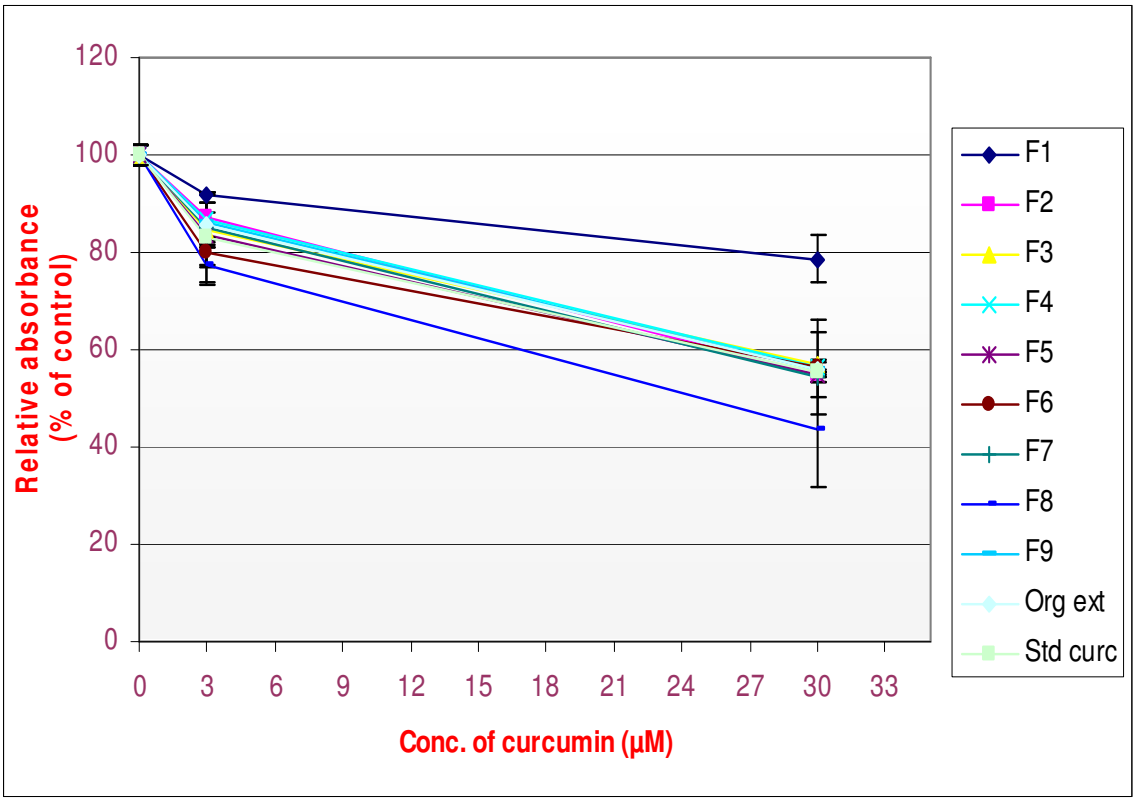


Figure 26 – *MCF-7 cell viability in the presence of different concentrations of turmeric fractions, original extract and standard curcumin*

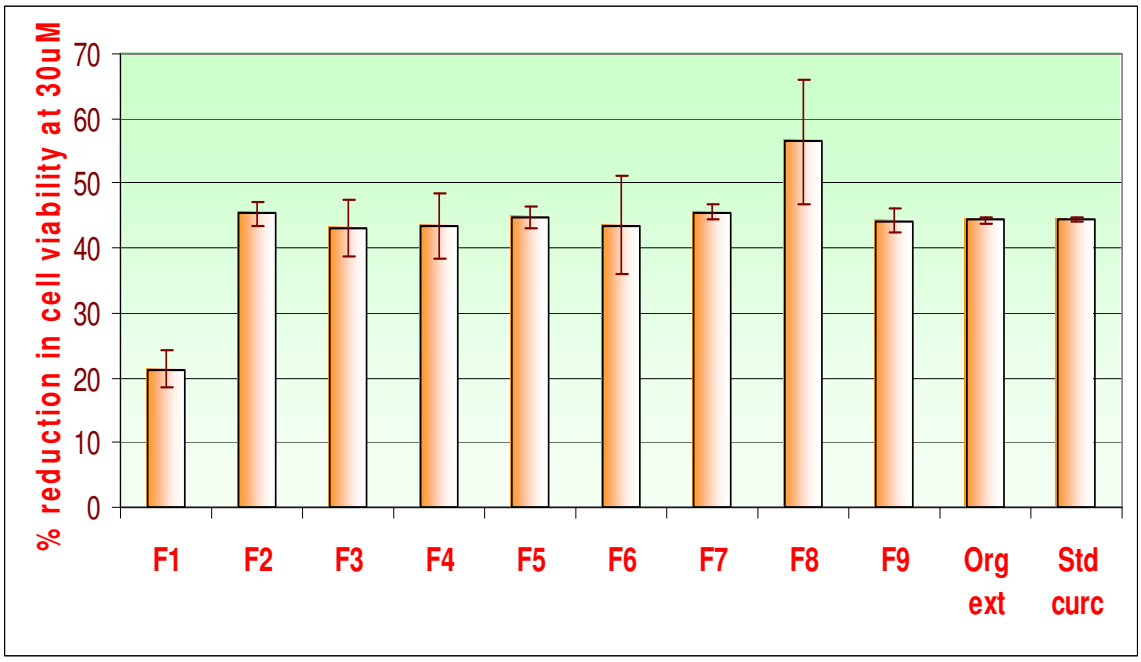


Figure 27 – *The percent reduction in MCF-7 cell viability at 30µM for all the turmeric fractions including the original turmeric extract and standard curcumin. % Cell viability reduction = [100 - (A_s – A_b)/A_v×100%] where A_s = Absorbance of test sample well (well with sample+cells+media), A_b = Absorbance of blank control well (well with sample + media but no cells), A_v = Absorbance of vehicle control well (well with cells+DMSO of 0.5%)*

CHAPTER IV

CONCLUSIONS AND FUTURE SCOPE OF THIS RESEARCH

One of the major goals of our research group is to determine how the complex mixtures of plant compounds contribute to the over all biological activity of the plant extracts. In the case of this research, an approach was developed that allowed for investigation of the contributions of multiple, potentially bioactive compounds to the anti-proliferative activity of *C. longa*. A simple and rapid HPLC-ESI-MS method was employed for the phytochemical characterization of extracts and fractions from this plant, and was successfully applied for both qualitative and quantitative analysis of several major constituents. A novel approach relying on the quantitative analysis of the fractions was then used to test these fractions for anti-proliferative activity against the MCF-7 human breast cancer cell line. With this approach, it was possible to quickly determine that curcumin alone was primarily responsible for the overall anti-proliferative activity of the complex turmeric extract. Some anti-proliferative activity was also observed for a fraction containing ar-tumerone and other volatile constituents, but these constituents did not appear to contribute significantly to the overall anti-proliferative activity of the complex *C. longa* extract. While specific synergistic or additive interactions were not identified for *C. longa* in this study, the novel approach developed here could certainly be employed in the future for the investigation of other medicinal plants with potentially synergistic or additive activity.

This is an exciting area of research which our research group has focused on. The main purpose of this study was to identify the isolated turmeric fractions that have shown significant *in vitro* anti-proliferative activity. This is not the end of this research. There is still more investigation that needs to be done. Further studies must be designed to gain deeper understanding of the actual content in the isolated turmeric fractions. Fractions that showed significant activity will be subjected to further isolation using flash chromatography and/or preparatory scale HPLC. Ultimately, we seek this experimental approach to determine which specific fractions of turmeric are responsible for its anti-proliferative activity. Once the isolated active components of the extract are identified, they have to be further studied using *Dictyostelium* cell model in order to gain more insight into the mechanism of action. Since the exact mechanism of anti-proliferative activity of curcumin is not clearly understood, the outcomes of this *Dictyostelium* cell assay would prove highly beneficial.

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